

Ulla-Britt Ericsson

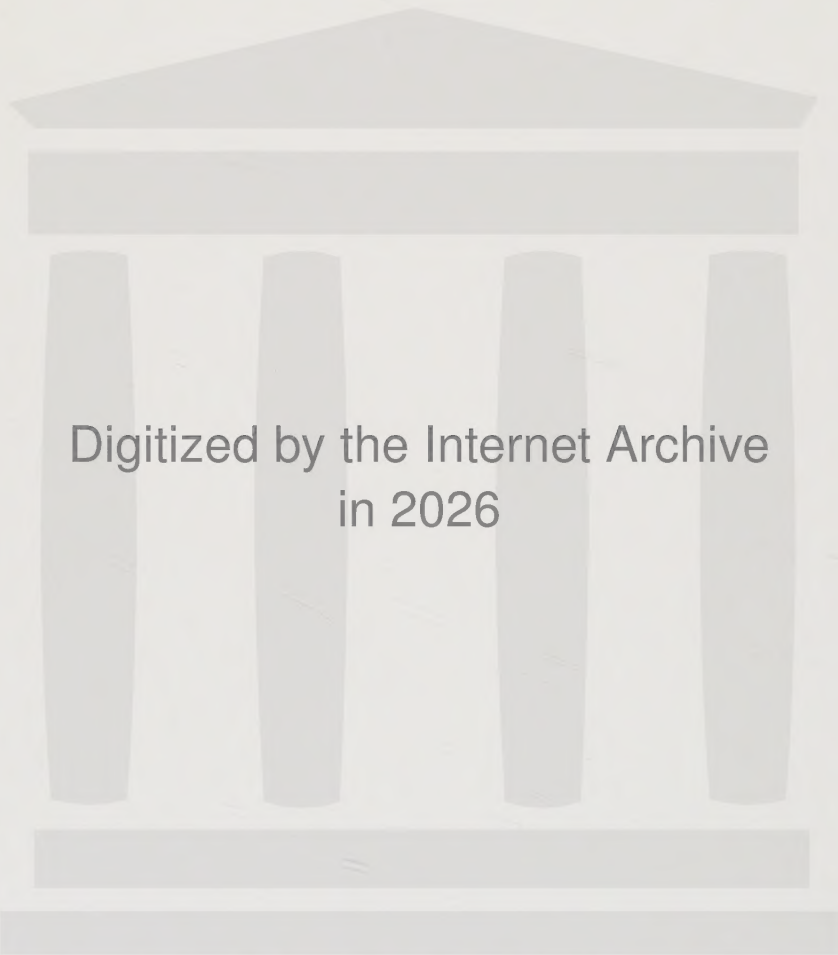
THYROGLOBULIN AND THYROGLOBULIN AUTOANTIBODIES

Methodological and clinical studies



Malmö 1984

Diss.
Rural.
1984



Digitized by the Internet Archive
in 2026

https://archive.org/details/IA41546724_0058

Organization LUND UNIVERSITY	Document name DOCTORAL DISSERTATION	
	Date of issue 1984-09-28	
	CODEN: LUMEDW-(MEMM-1017)/1-115(1984)	
Author(s) Ulla-Britt Ericsson	Sponsoring organization	
Title and subtitle		
Thyroglobulin and thyroglobulin autoantibodies. Methodological and clinical studies.		
Abstract Factors that could influence the stability of thyroglobulin (Tg) were studied as well as the effect of unstable Tg on the results in a double-antibody radioimmunoassay for Tg. Purification of Tg without the addition of protease inhibitors and freezing of the purified Tg in buffer resulted in unstable Tg. With the addition of protease inhibitors during the purification procedure and by storage of the purified Tg in 50% glycerol or goat serum at -20°C the Tg remained in its native form. The effects of the utilization of unstable Tg as standard and tracer in the radioimmunoassay for Tg were dependent on the quality of the antiserum and with certain antisera resulted in a gradual loss of immunoreactivity. A sensitive solid-phase immunosorbent radioassay for the detection of antibodies to polypeptide hormones and proteins is described. When this assay was used for the detection of Tg autoantibodies, these were demonstrated in 36% of the healthy women and in 15% of the healthy men. Interference of endogenous lactoperoxidase antibodies, which are present in most healthy individuals, was demonstrated in the assay, when iodination with the lactoperoxidase method was used. The clinical studies evaluated the usefulness of serum Tg (S-Tg) determination in the follow-up of patients with differentiated thyroid carcinoma after ablative therapy and as a method to predict the risk of relapse in patients with thyrotoxicosis. In thyroid carcinoma patients measurement of S-Tg during thyroxine treatment correlated well with the results of radioiodine scanning. In patients with a S-Tg concentration below 10 µg/l, no radioiodine scan is required. In patients with thyrotoxicosis S-Tg determination is of limited value in predicting the risk of relapse.		
Key words		
Thyroglobulin, thyroglobulin autoantibodies, radioimmunoassay, purification procedures, thyroid carcinoma, thyrotoxicosis, thyroiditis, lactoperoxidase antibodies		
Classification system and/or index terms (if any)		
Supplementary bibliographical information		Language English
ISSN and key title		ISSN
Recipient's notes	Number of pages 115	Price
	Security classification	

Distribution by (name and address) Ulla-Britt Ericsson, Department of Medicine, Malmö General Hospital, S-214 01 Malmö, Sweden

I, the undersigned, being the copyright owner of the abstract of the above-mentioned dissertation, hereby grant to all reference sources permission to publish and disseminate the abstract of the above-mentioned dissertation.

Signature Ulla-Britt Ericsson

Date 1984-07-30

THYROGLOBULIN AND THYROGLOBULIN AUTOANTIBODIES

Methodological and clinical studies

AKADEMISK AVHANDLING

som med vederbörligt tillstånd av Medicinska fakulteten vid Lunds
Universitet för avläggande av medicine doktorsexamen kommer att
offentligen försvaras i Medicinska klinikens aula, Allmänna Sjuk-
huset, Malmö, fredagen den 28 september 1984 kl. 9.00

av

Ulla-Britt Ericsson

med lic, mlm

THYROGLOBULIN AND THYROGLOBULIN AUTOANTIBODIES
Methodological and clinical studies

By

Ulla-Britt Ericsson

Department of Internal Medicine and Section of Nuclear Medicine at
the Department of Clinical Chemistry, University of Lund,
Malmö General Hospital, Malmö, Sweden

Malmö 1984

This dissertation is based on the following papers, which will be referred to in the text by Roman numerals I-VII:

- I. U-B. Ericsson, I. Larsson and J.I. Thorell. Purification and storage of thyroglobulin. Two important factors influencing the radioimmunoassay for thyroglobulin. Scand J Clin Lab Invest. In press.
- II. U-B. Ericsson, I. Larsson, A. Murne and J.I. Thorell. A new sensitive immunosorbent radioassay for the detection of circulating antibodies to polypeptide hormones and proteins. Scand J Clin Lab Invest. In press.
- III. U-B. Ericsson and I. Larsson. Interference of endogenous lactoperoxidase antibodies in a solid-phase immunosorbent radioassay for antibodies to protein hormones. Accepted for publication in Clin Chem.
- IV. U-B. Ericsson, L. Tegler, S. Lennquist, S. Borup Christensen, E. Ståhl and J.I. Thorell. Serum thyroglobulin in differentiated thyroid carcinoma. Acta Chir Scand 150:367-375, 1984.
- V. O. Ljungberg, U-B. Ericsson, L. Bondeson and J.I. Thorell. A compound follicular-parafollicular cell carcinoma of the thyroid: A new tumor entity? Cancer 52:1053-1061, 1983.
- VI. U-B. Ericsson, L. Tegler, J.F. Dymling and J.I. Thorell. Effect of therapy on the serum thyroglobulin concentration in patients with toxic diffuse goitre, toxic nodular goitre and toxic adenoma. Submitted for publication.
- VII. U-B. Ericsson, S. Borup Christensen and J.I. Thorell. The prevalence of thyroglobulin antibodies in adults with and without thyroid disease as measured with a sensitive solid-phase immunosorbent radioassay. Submitted for publication.

Cover by Lars Jansson

Mwĩigananio ti wa mweri

To obtain what one wants is not a month's job

(A kikuyu proverb)

CONTENTS

ABBREVIATIONS	6	
INTRODUCTION	7	
AIMS OF THE STUDY	11	
RESULTS AND COMMENTS		
<i>METHODOLOGY</i>		
Purification of thyroglobulin	12	
Storage of thyroglobulin	12	
Thyroglobulin radioimmunoassay	13	
Antibody detection	15	
Interference of endogenous antibodies to bovine lactoperoxidase	16	
<i>CLINICAL STUDIES</i>		
Identification of a new tumour entity?	18	
Serum thyroglobulin in differentiated thyroid carcinoma	19	
Serum thyroglobulin in thyrotoxicosis	21	
The prevalence of thyroglobulin autoantibodies	23	
GENERAL SUMMARY	27	
ACKNOWLEDGEMENTS	29	
REFERENCES	30	
ARTICLE	I	45
"	II	55
"	III	63
"	IV	71
"	V	81
"	VI	91
"	VII	105

ABBREVIATIONS

Tg	= Thyroglobulin
S-Tg	= Serum thyroglobulin
Tg-ab	= Thyroglobulin autoantibodies
PI	= Protease inhibitors
Tg-PI	= Thyroglobulin purified protected against the influence of protease inhibitors
Tg-O	= Thyroglobulin purified unprotected against the influence of protease inhibitors
Tg-vH	= Thyroglobulin, a gift from Dr van Herle, purified unprotected against the influence of protease inhibitors
LPO	= Lactoperoxidase
RIA	= Radioimmunoassay
DA	= Double-antibody radioimmunoassay
DASP	= Double-antibody solid-phase immunosorbent radioassay
TRC	= Tanned red cell haemagglutination test
LATS	= Long acting thyroid stimulator
TSI	= Thyroid stimulating immunoglobulins

INTRODUCTION

Thyroglobulin (Tg) is the main protein synthesized in the follicle cell of the thyroid gland. It is the key precursor in the biosynthesis of thyroid hormones and usually accounts for 75% of the total protein content of the mammalian thyroid (130). Tg was named in 1899 by Hutchison (94). Since then it has been subject to a great number of investigations, especially during the last 20 years. It is a large globular glycoprotein with a molecular weight of 660 000 daltons and a sedimentation constant of 19S (46), composed of two polypeptide subunits, each with a molecular weight of about 300 000 daltons (12S Tg) (5,151,153). The Tg molecule contains about 5500 amino acids (133), which are linked to peptide chains on the rough endoplasmic reticulum of the follicle cell (22,48,91,124). Human Tg contains three types of carbohydrate units which contribute to about 10% of the weight of the Tg molecule (6,135,151).

The amount of iodide incorporated into the Tg molecule is dependent on the iodine supply and therefore may vary over a wide range in mammalian thyroid glands i.e. between 10 and 50 atoms of iodide per mole are distributed among the 140 tyrosyl residues present in each Tg molecule (86,112,124,140).

Tg serves as the backbone in the synthesis of the thyroid hormones thyroxine (T_4) and triiodothyronine (T_3). It should be regarded as a prohormone because, after intracellular proteolysis, metabolically active iodothyronines are released into the circulation.

For several decades Tg was thought to be confined to the thyroid gland. In 1967 Daniel et al. (40) demonstrated with the use of a radioimmunoassay for Tg, the presence of Tg in the cervical lymphatics of monkeys and later iodoproteins were also demonstrated in the cervical lymphatics of rats (41). These studies established one route of release of Tg into the circulation but did not clarify what fraction of Tg is released via the lymphatic pathway (121) and other possible pathways in different thyroid diseases were not evaluated.

The presence of Tg in the circulation was first definitely demonstrated by Roitt and Torrigiani in 1967 (108) with a radioimmunoassay for Tg. With the subsequent development of more clinically practicable and sensitive radioimmunoassays, Tg was found in the circulation of 74-86% of normal subjects (105).

The biochemical characterization of circulating Tg has offered problems because of the low serum concentration present in healthy individuals. The S-Tg has a decreased stability compared to Tg in the thyroid follicles suggesting that it contains less iodide (72, 121). The lower density of S-Tg compared to follicular Tg (38,118, 121,123) supports this suggestion. The low iodide content of Tg in serum indicates that Tg may be directly secreted from the follicle cell to the circulation without preceding secretion to and reuptake from the lumen of the follicle cell (121).

Tg is cleared from the circulation by the hepatic degradative pathway used in common by many glycoproteins (71). The half-life of the circulating Tg in different studies has varied from four hours to six days (52,74,84,121,139).

During physiological conditions TSH is probably the most important regulator of Tg release (24,142,144). Even in certain pathological conditions TSH or other thyroid stimulating proteins may increase Tg release. In the rat, long acting thyroid stimulator (LATS) has been shown to increase S-Tg, but the response is slower, more prolonged and less pronounced than with TSH (149). In Graves' disease, which is characterized by the presence of stimulating immunoglobulins, these may be responsible for the increased release of Tg into the circulation. In differentiated thyroid carcinoma, the release of Tg from the cancer tissue is also responsive to TSH (85,115,122).

However, in differentiated thyroid carcinoma and other thyroid disorders, TSH is not the only modulator of Tg release, since even during treatment with thyroxine, when TSH is suppressed, measurable amounts of Tg are sometimes present in the circulation.

Table I. Comparison of reference range and sensitivity between different radioimmunoassays for determination of serum thyroglobulin in man.

Authors	Reference range S-Tg µg/l	Sensitivity µg/l	Place
Torrighiani et al. 1969 (141)	10-150	10	London, England
van Herle et al. 1973 (148)	<1.6-20.7	1.6	California, USA
Pezzino et al. 1977 (101)	2.5-43	2.5	Pisa, Italy
Schneider et al. 1977 (119)	0.8-28	0.8	Chicago, USA
Feldt-Rasmussen et al. 1977 (51)	<1.7-32.6	1.7	Denmark
Shah et al. 1978 (126)	1.0-20	0.1	Bombay, India
Bodlaender et al. 1978 (25)	2-61	2	California, USA
Bayer et al. 1979 (15)	<40	2.5	California, USA
Endo et al. 1979 (49)	3.5-59.5	3.5	Japan
Schlumberger et al. 1979 (114)	<2.5-29.5	2.5	Villejuif, France
Gardner et al. 1979 (59)	<4-28	4	Philadelphia, USA
Ikekubo et al. 1980 (70)	4-25.7	4	Chicago, USA
Pacini et al. 1980 (98)	<1.25-27	1.25	Pisa, Italy
Charles et al. 1980 (35)	3.4-35.1	0.6	California, USA
Hüfner et al. 1980 (69)	5-120	5	Heidelberg, FRG
Benita et al. 1981 (19)	3.8-150	2	Nice, France
Dimitriu et al. 1981 (43)	4.67-37.05	1.5	Bucharest, Romania
Botsch et al. 1983 (29)	0-70	2	Berlin, FRG
Kawamura et al. 1983 (77)	<2.5-68	2.5	Osaka, Japan
Ericsson et al. 1984	<2-40	2	Malmö, Sweden

There is considerable variation in the normal range of S-Tg reported from different laboratories (Table I). These differences probably reflect the lack of standardization of the assays, especially with respect to the handling of the rather fragile Tg molecule. As seen from Table I, the wide normal range found with certain assays is not merely due to their lack of sensitivity. Neither could these discrepancies be ascribed to true differences in the populations studied as no true geographic variation can be shown.

Increased levels of Tg in serum have been reported in various benign and malignant thyroid disorders including toxic goitre, non-toxic goitre, subacute thyroiditis and differentiated thyroid carcinoma (53,61,101,152).

In 1969, Torrighiani et al. (141) described a patient with thyroid cancer whose elevated S-Tg was normalized after surgical removal of

the tumour and later became undetectable. This observation was followed by more extensive studies in patients with differentiated thyroid carcinoma (12,18,28,29,35,63,69,75,83,90,97,113,117,119,127,129,150). There is, however, disagreement about the clinical value of S-Tg measurement in comparison with the usually used radioiodine scan in the follow-up of these patients after surgery (7,17,33,37,45,58,87,98,122,138).

Elevated S-Tg has also been found in most patients with thyrotoxicosis (44,49,53,101,141,148). The finding by van Herle et al., that LATS stimulated the release of Tg into the circulation, supports the concept that TSI are responsible for the increased S-Tg concentrations found in patients with Graves' disease (149). From the results of a retrospective study in patients with Graves' disease, Uller et al. (143) suggested that S-Tg determination might be a valuable indicator of remission in thyrotoxicosis. However, the usefulness of S-Tg determinations in this respect has not been confirmed by others (54) and is therefore still open for debate.

Thyroglobulin autoantibodies (Tg-ab) were first demonstrated in the serum of patients with Hashimoto's disease by Roitt et al (106). At that time Tg was considered to be a seclused antigen, which only in pathological conditions reached the circulation, giving rise to the formation of Tg-ab (95). This concept was later challenged when Tg was shown to be a normal secretory product from the thyroid gland (8,68). The development of sensitive RIA methods for the measurement of Tg-ab has further increased the knowledge within the field of autoimmune thyroid diseases. However, the clinical and pathological significance of the Tg-ab in apparently healthy individuals remains unclear.

AIMS OF THE STUDY

The aims of the present study were

1. to evaluate the influence of technical factors, such as variations in the purification procedure of Tg and in the stability of Tg, on the results in a double-antibody RIA for Tg
2. to study the clinical usefulness of S-Tg determination, in particular in comparison with the usually used radioiodine scan in the follow-up of patients with differentiated thyroid carcinoma after total thyroidectomy and as an indicator of remission in thyrotoxicosis
3. to develop an improved sensitive radioassay generally applicable for detection of autoantibodies to polypeptide hormones and proteins and to study its application for the assay of Tg-ab
4. to re-evaluate the prevalence of Tg-ab in healthy individuals and in patients with different thyroid disorders.

RESULTS AND COMMENTS

METHODOLOGY

Purification of thyroglobulin (I)

Endogenous proteolytic activity has been found in several purified Tg preparations (103,109,134). Tg isolated by different procedures was still found to be contaminated by endogenous proteases of both acid and alkaline pH optima (109). These contaminating endogenous proteases were thought to be involved in the degradation of the native 19S Tg (103,109). Despite the addition of different protease inhibitors (from one to four in different combinations) heterogeneity of the Tg preparations remained (47,103,109,147). In order to obtain a more complete inhibition of the proteolytic activity, we therefore used a combination of eight potent protease inhibitors in the primary purification step. In the presence of these protease inhibitors, Tg eluted as one dominating peak, with less than one third of the proteins emerging as side fractions of higher or lower molecular size, while more than 50% of the Tg, purified without the protection from protease inhibitors, appeared in fractions of lower molecular size. Thus, despite the protection by a broad spectrum of protease inhibitors, some heterogeneity remained, although it was much less than without this protection. So far no valid explanation has been given to this always prevailing heterogeneity, but it has been hypothesized that the enzyme may constitute an inherent part of the molecule or that a proteolytic enzyme may be irreversibly bound to the molecule, leading to a continuous degradation of the protein (3).

Storage of thyroglobulin (I)

The effect of freezing in buffer and 50% glycerol on Tg purified with (Tg-PI) and without (Tg-0) the protection of protease inhibitors, was compared. Freshly iodinated Tg from the two Tg preparations both contained mainly 19S Tg (660 000 daltons, Fig. 2 in article I). After freezing-thawing once in buffer, almost all Tg-0 had dissociated into 12S protein (330 000 daltons) while about 50% of the Tg-PI remained as 19S Tg (Fig. 3 in article I). Freezing in

50% glycerol had no adverse effect on Tg-PI, while Tg-0 showed increased fractions of smaller size proteins (Fig.4 in article I). Thus, it seems that by protecting the Tg from influence of proteolytic enzymes, a more stable Tg preparation is obtained.

On freezing in buffer some proteins lose their native structure (80,136). During freezing in buffer many compositional changes occur simultaneously. Water is frozen out of solution leading to an increase in pH, salt concentration etc. (39,104,137).

Tg, especially Tg with low iodine content, is unstable (34,39,73,78, 79,131,132,145). On freezing-thawing in buffer solutions, 19S Tg has been found to dissociate into proteins of a smaller molecular size (131), as confirmed in this study. The degree of dissociation on freezing is dependent on the degree of iodination of the Tg molecule (73) as well as the pH and ionic strength of the buffer (5,39). According to van der Walt (147), the effect of freezing on Tg is more pronounced than the degradation by proteases. Addition of PMSF (phenyl-methyl-sulfonyl fluoride) to the buffer did not inhibit the degradation of Tg on freezing, whereas without the freezing there were fewer smaller size proteins. In contrast to van der Walt, however, we found that Tg protected by protease inhibitors was more stable than Tg unprotected from proteolytic activity. This effect was obvious on freezing in buffer as well as on freezing in 50% glycerol, which is known to protect proteins from the adverse effect of freezing (30,128). The difference between these two studies may be explained by species differences in the susceptibility to freezing or that the use of only one protease inhibitor was insufficient for the inhibition of the proteolytic activity.

Thyroglobulin radioimmunoassay (I)

Three different Tg preparations (Tg-PI, Tg-0 and Tg-vH) were used in a double-antibody radioimmunoassay both as tracer and as standard. Tg-PI and Tg-vH were also used for antisera production. Several distinct differences between these Tg preparations were observed.

Using the same iodination procedure, the incorporation of ^{125}I into the Tg-PI was 70-80% (specific activity 1.30-1.48 MBq/ μg), while the corresponding values for Tg-O and Tg-vH were 20-50% (specific activity 0.37-0.93 MBq/ μg). The use of Tg-PI in the RIA always gave a high maximal binding (B_0) which was only slightly reduced after freezing the tracer in buffer, while Tg-O gave a lower maximal binding, which was further reduced after freezing in buffer. After freezing ^{125}I -Tg-PI and ^{125}I -Tg-O in 50% glycerol the maximal binding was almost unchanged (Table II).

On freezing the stock standard in buffer a progressive loss of immunoreactivity with time was observed. After three months the apparent Tg concentration was doubled when read against this standard, and after five years it had increased five-fold (Fig. 5 in article I). A stock standard frozen in normal goat serum or 50% glycerol did not show any tendency to lose potency even after one year (Figs. 1 and 2).

The apparent loss of immunoreactivity of the Tg standard during storage in buffer at -20°C varied with different antisera (Fig. 5 and 7 in article I). The loss of immunoreactivity was apparent with antiserum k-7502, but did not occur with antiserum k-8102. One possible explanation for this variation is a difference in the quality of the antisera. Antibodies to native globular proteins are directed mainly against conformational rather than sequential determinants (81, 125). For Tg the conformational antibodies have been shown to dominate (88). Therefore, the gradual loss of immunoreactivity during freezing in buffer may depend on a decrease in the number of such conformational antigenic determinants.

All these factors found to influence the final results in the RIA must be taken into ac-

Table II. Influence of protease inhibitors and storage mode on the binding in the radioimmunoassay.

Storage mode	Tg-PI $B_0\%$	Tg-P0 $B_0\%$
Unfrozen	67	54
Frozen-Buffer at -20°C	62	23
Frozen-Glycerol at -20°C	64	49

count when comparing results from different laboratories. Future work must therefore be directed towards obtaining standardization of these procedures. Until this is achieved it will be difficult to compare results from different laboratories.

Antibody detection (II, VII)

One of the main problems in radioimmunoassay of endogenous peptides is the interference of autoantibodies which prevents the accurate determination of the antigen. This error was first described by Berson and Yalow in their insulin RIA as early as in 1956 (20), but has later also been recognized for several other peptide hormones and proteins including Tg (16, 57,102,110,120).

Despite the long time since the first recognition of this problem, there is still no clinically useful method which has solved it.

The method described in paper II and VII is a solid-phase immunosorbent radioassay based on the binding of the homologous ^{125}I -labelled antigen to the antibodies which are then bound to anti-IgG antibodies covalently coupled to Sepharose. This semi-quantitative assay can easily be applied as a com-

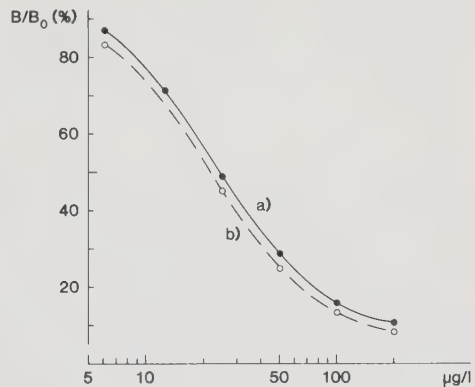


Figure 1. Dose-response curves for TgVH prepared from a stock standard frozen in normal goat serum using antiserum k-7502.

a. Stock standard unfrozen

b. Stock standard frozen at -20°C for one year

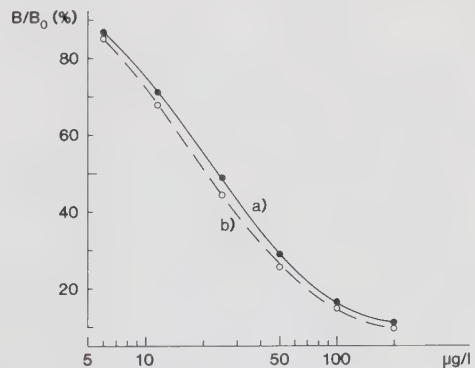


Figure 2. Dose-response curves for TgVH prepared from a stock standard in 50% glycerol using antiserum k-7502.

a. Stock standard unfrozen

b. Stock standard frozen at -20°C for one year

plement to any RIA for detection of circulating antibodies to the ligand measured by just applying the appropriate labelled antigen according to the antibody of interest.

Assays of Tg-ab based on a competitive binding RIA are likely to produce either falsely elevated or falsely depressed values in the presence of elevated S-Tg concentration (16). An advantage of the assay described in paper II apart from its wide applicability, is that false positive results due to interference of high serum concentrations of Tg, are avoided. False negative results, on the other hand, may occur in the presence of high serum concentrations of the antigen.

When this assay was applied to detect autoantibodies to Tg in a healthy control group, we found a higher frequency of Tg-ab positive sera, than had been described previously. Comparison with other Tg-ab RIA revealed differences in several respects which may influence the sensitivity of the assay. In Table III data from six different Tg-ab assays are summarized. The possibility to work with a low serum dilution and the use of a ten times higher specific activity of the tracer renders this assay more sensitive, thus enabling us to detect a higher proportion of the Tg-ab than has been possible before.

Interference of endogenous antibodies to bovine lactoperoxidase (III)

All human sera contain an immense number of antigens and antibodies. The performance of radioimmunoassays for the measurement of specific antigens and antibodies therefore demands that the presence of contaminating proteins in the antigen used in the assay is minimal. Interference of endogenous antibodies to insulin, Tg or TSH, in the RIA of these proteins, is a well known phenomenon (16,20,57,102,110, 120). During the development of a solid-phase immunosorbent radioassay for protein hormone antibodies unforeseen interference was recognized, which was found to be due to contamination of the tracer with self-iodinated bovine lactoperoxidase (LPO) used in the labelling procedure in combination with the frequent occurrence of endo-

Table III. Comparison between some factors which influence the sensitivity of double-antibody radioimmunoassays for measurement of thyroglobulin autoantibodies.

Authors	Separation method	Serum dilution in the primary incubate	Specific activity of thyroglobulin MBq/ μ g(mCi/mg)	Added labelled thyroglobulin ng	Concentration of labelled thyroglobulin in the incubate μ g/l
Salabè et al. 1974 (111)	DA	1:12 (120 μ l)	18.5 (0.5)	20	167
Peake et al. 1974 (99)	DA	1:100 (200 μ l)	111-148 (3-4)	1.5	7.5
Bodlaender et al. 1978 (25)	DA	1:21 (210 μ l)	-	2	9.5
Date et al. 1980 (42)	DA	1:30 (150 μ l)	7.4-18.5 (0.2-0.5)	9	60.000
Beall et al. 1982 (17)	DA	1:100 (200 μ l)	111-148 (3-4)	2-6	10-30
Ericsson et al. 1984	DASP	1:5 (500 μ l)	1110-1480 (30-40)	0.3	0.6

genous antibodies to bovine LPO in human sera. This error may occur in any assay system, in which the separation method precipitates the human IgG with the antibodies, including radioassays in which anti-IgG antibodies are used. In double-antibody radioimmunoassays (DA), where rabbit immunoglobulin is used as the precipitating agent, endogenous LPO antibodies will not affect the results.

Thus, when iodination with the LPO method is used, this previously unrecognized source of error must be borne in mind. This does not mean, however, that other labelling procedures should be preferred as the interference of LPO antibodies can easily be avoided by the use of purification procedures other than gel filtration for the tracer, such as high performance liquid chromatography or adsorption chromatography on cellulose or by the use of immobilized LPO.

CLINICAL STUDIES

Identification of a new tumour entity? (V)

An unusual thyroid carcinoma is described which shows structural, histochemical and radioimmunological features of both a follicular and parafollicular cell carcinoma. After total thyroidectomy, pulmonary metastases showed uptake of radioiodine and high serum concentration of Tg was found. Extracts from metastatic tumour tissue contained Tg but not calcitonin. Immunoreactive Tg, characteristic of carcinomas of follicular origin, was demonstrated histochemically. Also somatostatin and neurotensin immunoreactivity, characteristic of cells of parafollicular origin (10,21,157), was demonstrated and in some tumour cells this occurred together with Tg. Light microscopy showed cribiform patterns suggestive of a follicular carcinoma as well as solid areas reminiscent of a medullary carcinoma. Electron microscopy revealed two types of tumour cells, one with dense granules resembling granules characteristic of polypeptide hormone and/or monoamine producing endocrine cells and the other without granules but with a vesicular rough endoplasmic reticulum similar to that seen in neoplastic follicular cells.

These results indicate that this tumour is a structurally and functionally biphasic tumour which contains elements of both follicular

and parafollicular cells. It resembles a case reported by Bussolati and Monga (31) as an "atypical medullary carcinoma" which showed light microscopic, histochemical and biochemical features consistent with a calcitonin-producing, amyloid-rich medullary carcinoma, but with atypical microfollicular structures and electron microscopic patterns reminiscent of a follicular carcinoma. In their case, the presence of Tg in the tumour or in the circulation was not investigated. A similar tumour but with no calcitonin secretion (as in the case described by us) has been described by Valenta et al. (146). Recently, Hales and coworkers (64) have described a thyroid carcinoma with a predominantly medullary pattern admixed with areas of follicular differentiation. Calcitonin as well as Tg was demonstrated in the neoplastic cells, but it was not clarified whether they occurred in the same tumour cells. This tumour was also shown to concentrate radioactive iodine.

The finding of both Tg and somatostatin or neurotensin immunoreactivity together in some tumour cells questions the current belief that follicular and parafollicular cells are derived from two functionally and embryogenetically different cell types, entoderm and neuroectoderm respectively. Instead, a common stem cell may be the progenitor of both these cell types.

Serum thyroglobulin in differentiated thyroid carcinoma (IV)

S-Tg concentrations were measured in 262 patients with differentiated thyroid carcinoma (70 men and 192 women). S-Tg was measured before treatment in 82 patients and after treatment in 240 individuals. The thyroid carcinomas were classified according to WHO 1974 (65). Follicular carcinoma was present in 106 subjects and classified as Hürthle cell carcinoma in 15 of them. A diagnosis of papillary carcinoma was made in 135 patients and medullary carcinoma was found in 21 subjects.

Analysis of S-Tg with respect to the histological classification showed that the follicular variety of carcinoma had the highest S-Tg concentrations pre- as well as postoperatively when metastases were found, which is in accordance with what has been found by others

(63, 127). The Hürthle cell carcinomas behaved in this respect as the follicular carcinomas despite the fact that Hürthle cell adenomas or carcinomas have been said not to synthesize Tg (66) or to be nearly devoid of Tg (32).

The wide variation found in the secretion pattern of the papillary carcinomas has also been observed by others (116,127,129) and may be due to different proportions of follicular areas within the tumours. The pure papillary carcinomas had the lowest preoperative S-Tg concentrations which is in accordance with this assumption.

Patients with medullary carcinomas had the lowest preoperative S-Tg concentrations. After total thyroidectomy all three patients with metastases had S-Tg levels below 10 µg/l. This is in agreement with the lack of Tg synthesis in medullary carcinomas demonstrated by Ljunggren et al. (82). Thus S-Tg measurement is of no value in the follow-up of these patients.

Like Black et al. (23) and Feldt-Rasmussen et al. (55) we found the S-Tg determination to be equally useful in patients with and without Tg-ab. On the other hand, presence of residual thyroid bed tissue decreased the diagnostic potential of the S-Tg as has also been stated by Schlumberger et al. (116).

It has been disputed whether the S-Tg should be measured during replacement therapy or after induction of hypothyroidism (7,37,116, 122). Our results indicate that the S-Tg concentration during thyroxine treatment accurately predicts the results of the ¹³¹I total body scan in most patients. In a review in 1982, Charles (36) stated that the additional diagnostic efficiency of performing S-Tg measurements in hypothyroid patients may not justify the negative effects of inducing hypothyroidism.

The usefulness of S-Tg measurements in relation to total body iodine scan in the follow-up after ablative therapy, has also been subject to much dispute. The conclusion of the present study is that the determination of S-Tg can replace total body iodine scan in the fol-

low-up of patients with differentiated thyroid carcinoma (except medullary) after ablative therapy. On the basis of cumulative data on 1323 reported cases of well-differentiated thyroid cancer comparing Tg and total body iodine scan, Ashcraft and van Herle (7) conclude that Tg is a more sensitive screening device than the scan in the early detection of metastases after treatment of thyroid cancer. From a review of the literature Charles (36) concludes that the S-Tg determination could replace the radioiodine scan in patients with thyroid cancer with remarkable confidence, reduced morbidity, reduced costs and less patient and physician time expenditure.

From the combined results of our study and the literature the following scheme for the treatment and follow-up of patients with differentiated thyroid carcinoma is proposed. After total thyroidectomy a postoperative scan is performed. Residual functional thyroid tissue is eradicated with ^{131}I and the effect of the treatment is evaluated with a new scan after 3 months. Patients with negative scans are thereafter followed with repeated S-Tg determinations during thyroxine treatment once or twice a year. Only when the S-Tg concentration rises above 10 $\mu\text{g/l}$ is a total body iodine scan required.

Serum thyroglobulin in thyrotoxicosis (VI)

S-Tg is elevated in the majority of the patients with thyrotoxicosis irrespective of the pathogenesis (44,49,53,61,93,101,141,148). In Graves' disease the TSI are thought to stimulate the release of Tg into the circulation (149), whereas in toxic nodular goitre and toxic adenoma, the mechanism(s) behind the increased secretion of Tg is unclear.

On the basis of the results of a retrospective study Uller and van Herle (143) proposed that the mean S-Tg at the end of therapy could be a useful index of the eventual outcome in patients treated with anti-thyroid drugs.

In order to test the validity of this hypothesis in a prospective study and to evaluate the usefulness of S-Tg measurements in pa-

tients with toxic nodular goitre and toxic adenoma, 38 patients with Graves' disease, 19 with toxic nodular goitre and 7 with toxic adenoma were followed with repeated measurements of S-Tg during the course of therapy with anti-thyroid drugs and before and after treatment with radioiodine and surgery.

In agreement with previous results (44,49,53,93,101,141,148) we found elevated S-Tg concentrations in most patients with thyrotoxicosis. Unlike Uller et al. (143) we observed an overlap in S-Tg concentration between normal and hyperthyroid subjects, being most pronounced in the patients with Graves' disease (Fig 1. in article VI). The pre-treatment S-Tg concentration was significantly higher in patients with toxic nodular goitre (median value 79 $\mu\text{g/l}$) and toxic adenoma (median value 70 $\mu\text{g/l}$) than in patients with toxic diffuse goitre (median value 49 $\mu\text{g/l}$).

Like Pezzino et al. (101) we did not find any significant correlation between the pre-treatment S-Tg concentration and the T_3 or T_4 concentration in any of the types of thyrotoxicosis. Thus, there seem to be no direct relation between the S-Tg concentration and the severity of the disease.

In the 12 patients with Graves' disease treated with anti-thyroid drugs (in combination with thyroxine), we found a variable course of the S-Tg concentration irrespective of whether they continued in remission or had a relapse after stopping anti-thyroid drug treatment (Figs. 2-4 in article VI). No correlation was observed between the course of the S-Tg concentration and the outcome of therapy, but there was a tendency towards higher pre-treatment S-Tg values in those patients who later had an exacerbation, which has also been observed by Uller et al. (143) and Gardner et al. (59).

The lack of correlation between the S-Tg concentration and the outcome of therapy may be due to the fact that the number of individuals in the study groups are too few. However, a similar variation in the course of S-Tg as observed by us, has also been seen in most previous studies (77,101,143), but the proportion of patients

with declining or increasing S-Tg concentrations has varied. This variation may explain the different conclusions drawn from these studies.

In patients with Graves' disease and toxic nodular goitre treated with radioiodine, the same type of varying individual S-Tg patterns was observed. No significant difference was found in the mean S-Tg concentration between the patients in remission and those who had an exacerbation.

In patients with thyrotoxicosis irrespective of type, treatment with surgery was followed by a decrease in S-Tg concentration and only 1/23 patients had a relapse.

The prevalence of thyroglobulin autoantibodies (VII)

The prevalence of Tg-ab was estimated in a total of 368 individuals (271 women and 97 men), of whom 140 (80 women and 60 men) constituted a healthy control group without a history of thyroid disease and without clinical or biochemical evidence of thyroid disease. In all individuals, Tg-ab were measured with RIA technique and with passive haemagglutination (TRC). In all groups a higher frequency of Tg-ab was detected with the RIA than with the TRC method (Table II in article VII). In the control group, Tg-ab were found in 27% of the individuals (36% of the women and 15% of the men) as measured with the RIA technique. A comparable high frequency has only been reported by Pinchera et al. (102) who found Tg-ab in 32.4% of the controls (Table IV). But in their radioassay false positive results may occur, due to interference of high serum concentrations of Tg. In our radioassay, however, false positive results due to high serum concentrations of Tg do not occur, but high S-Tg concentrations may give rise to false negative results.

All patients with primary hypothyroidism were Tg-ab positive with the RIA, whereas only 56% were positive with the TRC test. Tg-ab were found in 81% of the patients with toxic diffuse goitre, and in 30-40% of the patients with toxic nodular goitre, toxic adenoma,

non-toxic goitre and thyroid carcinoma. These results are in accordance with those found by others using sensitive RIA methods (89,99). With the TRC test, Tg-ab were positive only in 10-17% of these patients.

There is considerable variation in the reported frequency of Tg-ab in healthy individuals, which is evident from the results listed in Table IV. These discrepancies may be due to variation in sensitivity of the techniques of detection, in the definition of significant titres, in differences in the selection of the individuals composing the study groups as well as in true geographic differences in the prevalence of thyroiditis.

In a few previous studies the results from the TRC and the RIA methods have been compared (56,102). As in the present study, great

Table IV. Thyroglobulin autoantibodies in the serum of adults without thyroid disease as measured with the tanned red cell haemagglutination technique (TRC) or radioimmunological methods (RIA).

Authors	Methods	n	Detectable thyro- globulin antibodies %
Abreau et al. 1977 (1)	TRC	85	2
Borup Christensen et al. 1984 (27)	TRC	441 (F)	3.5
Kallifronas et al. 1981 (76)	TRC	573 (M+F)	4.1
Roitt and Doniach 1958 (107)	TRC	146 (M+F)	5.5
Pinchera et al. 1977 (102)	TRC	195	5.6
Hill et al. 1961 (67)	TRC	1297 (M+F)	5.7
Owen and Smart 1958 (96)	TRC	52 (M+F)	6
Althaus et al. 1983 (2)	TRC	91 (F)	6.5
Whittingham et al. 1969 (154)	TRC	575 (M+F)	6.9
Bastenie et al. 1967 (13)	TRC	785 (M+F)	12
Nève et al. 1966 (87)	TRC	430 (F)	16
Anderson et al. 1967 (4)	TRC	60	16.7
Salabè et al. 1974 (111)	TRC	57	17.5
Hackett et al. 1960 (62)	TRC	387 (M+F)	18
Salabè et al. 1974 (111)	RIA	57	1.7
Peake et al. 1974 (99)	RIA	25	4
Feldt-Rasmussen et al. 1983 (56)	RIA	197 (M+F)	6
Ericsson et al. 1984	RIA	140 (M+F)	27
Pinchera et al. 1977 (102)	RIA	195	32.8

discrepancies have been observed between the two methods. A total of 175 Tg-ab positive sera were found, of which 133 (76%) were positive with the RIA method, 7 (4%) with the TRC test and 35 (20%) were positive with both (Fig. 3). A comparable discrepancy has also been observed by Ewins et al. (50) and Feldt-Rasmussen et al. (56). No definite explanation for this discrepancy has been found, but it is probable that at least those sera with weak positive TRC titres may represent false positive results.

The clinical implication of these findings is that the TRC test has limited diagnostic value in thyroid diseases. On the other hand, the solid-phase immunosorbent radioassay may be too sensitive, giving low clinical specificity. Increased clinical specificity, however, is achieved by adjusting the definition of significant titre. Among the 27 patients with a histopathological diagnosis of thyroiditis (focal or diffuse) 25 had a titre of $\geq 1:50$ and 19 had a titre of $\geq 1:500$. Therefore a Tg-ab titre of $\geq 1:50$ should raise the suspicion of thyroiditis. The high prevalence of Tg-ab found in apparently healthy individuals is interesting and in agreement with fine needle biopsy and autopsy studies, where high frequencies of focal thyroid-

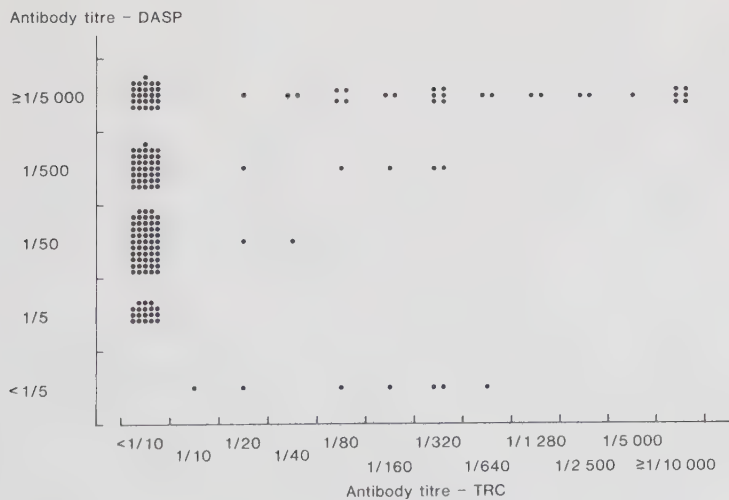


Figure 3. Comparison between the Tg-ab titre as measured with TRC and DASP in patients with detectable Tg-ab with either or both tests.

itis have been found (14,100,155,156). Williams et al. (155) found focal thyroiditis in almost 50% of the women 50 years old or above. Therefore, the frequency of Tg-ab observed may indicate a high prevalence of symptomless autoimmune thyroiditis. An alternative explanation would be that the Tg-ab of low titre in healthy individuals is a "physiological" phenomenon, which constitutes an alternative way for elimination of undegraded Tg in certain situations, for example when the normal degradation pathway is overloaded (60). This suggests that even more sensitive methods than the one used here would prove that most, if not all, "normal" sera contain small amounts of Tg-ab. In fact, already in 1965, Assem et al. (9), demonstrated by the use of electrophoretic immunorettention and chromatographic techniques, the presence of Tg-ab in more than half of the "normal" adult population and recently Bagnasco et al. (11) using an enzyme-linked immunosorbent radioassay, demonstrated anti-Tg autoantibodies of low concentrations in virtually all normal subjects.

GENERAL SUMMARY

Tg purified with protection from a broad spectrum of protease inhibitors was more stable than Tg purified without the addition of protease inhibitors.

Freezing-thawing of Tg in buffer was followed by dissociation of Tg into smaller size proteins. Tg purified in the presence of protease inhibitors was less susceptible to freezing in buffer than Tg purified without the addition of protease inhibitors.

Freezing in 50% glycerol or in goat serum had no adverse effect on the Tg.

On utilization of unstable Tg as standard and tracer in the radioimmunoassay for Tg a gradual loss of immunoreactivity of Tg was observed, resulting in a continuous drift in the concentration of a control serum read against this standard. The effect of unstable Tg on the results in the RIA was, however, also dependent on the quality of the antiserum.

A thyroid tumour is described demonstrating features of both a follicular and parafollicular cell carcinoma. No calcitonin was present in the tumour. High S-Tg was found and the pulmonary metastases accumulated radioiodine. Tg, neurotensin and somatostatin were demonstrated in the tumour cells, sometimes together in the same cells, supporting the possibility of a common precursor origin of both follicular and parafollicular cells.

S-Tg determination is a useful method for early detection of local recurrences in patients with differentiated thyroid carcinoma (except medullary) after total thyroidectomy. It can replace the radioiodine scan in those patients who have a S-Tg concentration below 10 $\mu\text{g/l}$. The S-Tg can be measured during thyroxine treatment, thereby reducing the total radioactive dose given to the patient and also the morbidity of induced hypothyroidism. The smaller personnel and

laboratory demands optimize the cost-benefit in the management of the patients.

Before treatment S-Tg was elevated in the majority of the patients with thyrotoxicosis, irrespective of pathogenesis. A tendency towards higher pre-treatment S-Tg concentrations was observed in the patients who subsequently relapsed. The post-treatment S-Tg concentration was of limited value in predicting relapse in thyrotoxicosis.

Endogenous antibodies to bovine LPO were demonstrated in the majority of healthy individuals. If iodination with the LPO-method is used, such antibodies can interfere with any assay system in which the separation method precipitates the human IgG with the antibodies, including radioassays where anti-IgG antibodies are used. In double antibody radioimmunoassays, where rabbit immunoglobulin is used as the precipitating agent, endogenous LPO antibodies will not affect the results.

With a sensitive immunosorbent radioassay Tg-ab were demonstrated in 36% of healthy women and 15% of healthy men. Whether these antibodies indicate the presence of a symptomless autoimmune thyroiditis or represents a "physiological" phenomenon remains unclear.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to all those who have helped and encouraged me throughout this study and especially to

Ass professor Jan Thorell, my supervisor, for introducing me into the field of radioimmunology, and for constructive criticism, and never failing support and enthusiasm

Ass professor Bertil Nosslin for providing laboratory facilities and for statistical advise and generous support

Ass professor Lennart Tegler and ass professor Sten Lennquist for stimulating cooperation, constructive criticism and invaluable help throughout the study

Mrs Alva Murne for excellent and enthusiastic laboratory assistance and generous support and encouragement

Ingvar Larsson and Lars-Erik Nilsson for performing the iodinations and for stimulating discussions

all other personnel at the Department of Nuclear Medicine whose work made this study possible

Inga-Lill Torp, Ann-Britt Nordvi and Lena Rubin for excellent secretarial aid with the final manuscript

Lars Jansson and Gunvor Nilsson for making the drawings

Britta Bernow, Eddie Fremer and Gun-Britt Lindqvist for professional help with the literature

my friends for constant support

REFERENCES

1. Abreau CM, Vagenakis AG, Roti E, and Braverman LE. Clinical evaluation of a hemagglutination method for microsomal and thyroglobulin antibodies in autoimmune thyroid disease. *Ann Clin Lab Sci* 7:73-78, 1977.
2. Althaus B, Staub JJ, Müller-Brand J, Carmann H, Viollier M, Matter L, und Berger W. Autoimmunantikörperprofil bei idiopathischer Hypothyreose und bei Hypothyreose nach Radiojodbehandlung wegen Morbus Basedow: Vergleich mit einem Normkollektiv aus der weiblichen Bevölkerung der Schweiz. *Schweiz Med Wochenschr* 113: 1319-1327, 1983.
3. Ambesi Impiombato FS, and Pitt-Rivers R. The nature of the breakdown of thyroglobulin. *FEBS Lett* 18:73-75, 1971.
4. Anderson JW, McConahey WM, Alarcón-Segovia D, Emslander RF, and Wakim KG. Diagnostic value of thyroid antibodies. *J Clin Endocrinol Metab* 27:937-944, 1967.
5. Andreoli M, Sena L, Edelhoch H, and Salvatore G. The noncovalent subunit structure of human thyroglobulin. *Arch Biochem Biophys* 134:242-248, 1969.
6. Arima T, Spiro MJ, and Spiro RG. Studies on the carbohydrate units of thyroglobulin. Evaluation of their microheterogeneity in the human and calf proteins. *J Biol Chem* 247:1825-1835, 1972.
7. Ashcraft MW, and van Herle AJ. The comparative value of serum thyroglobulin measurements and iodine 131 total body scans in the follow-up study of patients with treated differentiated thyroid cancer. *Am J Med* 71:806-814, 1981.
8. Assem ESK, and Cairo MB. Thyroglobulin in the serum of parturient women and newborn infants. *Lancet* i:139-141, 1964.
9. Assem ESK, Trotter WR, and Belyavin G. Thyroglobulin and thyroglobulin antibodies in the serum of normal adults. *Immunology* 9:21-29, 1965.
10. Bača I, and Schmidt-Gayk H. Neurotensin and medullary carcinoma of the thyroid gland. (Letter) *J Cancer Res Clin Oncol* 100:229-230, 1981.
11. Bagnasco M, Dirienzo W, Ferrini S, Ciprandi G, Merli A, and Melioli G. Anti-human thyroglobulin autoantibodies assayed by an enzyme-linked immunosorbent assay. *Ric Clin Lab* 13:247-254, 1983.
12. Barsano CP, Skosey C, DeGroot LJ, and Refetoff S. Serum thyroglobulin in the management of patients with thyroid cancer. *Arch Intern Med* 142:763-767, 1982.

13. Bastenie PA, Nève P, Bonnyns M, Vanhaelst L, and Chailly M. Clinical and pathological significance of asymptomatic atrophic thyroiditis. A condition of latent hypothyroidism. *Lancet* i:915-919, 1967.
14. Bastenie PA, and Ermans AM (eds). Thyroiditis and thyroid function. Clinical, morphological, and physiopathological studies. Pergamon Press, Oxford, 1972.
15. Bayer MF, and Kriss JP. Immunoradiometric assay for serum thyroglobulin: Semiquantitative measurement of thyroglobulin in antithyroglobulin-positive sera. *J Clin Endocrinol Metab* 49: 557-564, 1979.
16. Bayer MF, and Kriss JP. A solid phase, sandwich-type radioimmunoassay for antithyroglobulin: Elimination of false positive results and semiquantitative measurement of antithyroglobulin in the presence of elevated thyroglobulin. *J Clin Endocrinol Metab* 49:565-571, 1979.
17. Beall GN, Parslow ME, Kruger SR, and Zaidi A. Improved radioassay for human antithyroglobulin. *Acta Endocrinol (Copenh)* 101: 555-561, 1982.
18. Bednár J, Němec J, Zamrazil V, Váža S, Pohunková D, and Röhling S. Serum thyroglobulin determinations in patients with differentiated thyroid carcinoma. *Nuklearmedizin* 22:204-211, 1983.
19. Benita G, Lapalus F, Bayer V, Bornet H, and Hoch M. Radioimmunoassay of serum thyroglobulin. Technique and clinical results. *Eur J Nucl Med* 6:515-520, 1981.
20. Berson SA, Yalow RS, Bauman A, Rothschild MA, and Newerly K. Insulin- I^{131} metabolism in human subjects: Demonstration of insulin binding globulin in the circulation of insulin treated subjects. *J Clin Invest* 35:170-190, 1956.
21. Birnbaum RS, Muszynski M, and Roos BA. Tumor and plasma somatostatin-like immunoreactivity in transplantable rat medullary thyroid carcinoma. *Cancer Res* 40:4192-4196, 1980.
22. Björkman U, and Ekholm R. Thyroglobulin synthesis and intracellular transport studied in bovine thyroid slices. *J Ultrastruct Res* 45:231-253, 1973.
23. Black EG, Gimlette TMD, Maisey MN, Cassoni A, Harmer CL, Oates GD, and Hoffenberg R. Serum thyroglobulin in thyroid cancer. *Lancet* ii:443-445, 1981.
24. Black EG, Sheppard MC, and Hoffenberg R. Control of thyroglobulin secretion from the rat thyroid gland. *J Endocrinol* 101: 107-111, 1984.

25. Bodlaender P, Arjonilla JR, and Twomey SL. Sensitive radioimmunological screening test for anti-thyroglobulin autoantibodies. *Clin Chem* 24:272-274, 1978.
26. Bodlaender P, Arjonilla JR, Sweat R, and Twomey SL. A practical radioimmunoassay of thyroglobulin. *Clin Chem* 24:267-271, 1978.
27. Borup Christensen S, Ericsson U-B, Janzon L, Tibblin S, and Trell E. The prevalence of thyroid disorders in a middle-aged female population, with special reference to the solitary thyroid nodule. *Acta Chir Scand* 150:13-19, 1984.
28. Botsch H, Schulz E, and Lochner B. Serum-Thyreoglobulinbestimmung zur Verlaufskontrolle bei Schilddrüsenkarzinom-Patienten. *Dtsch Med Wochenschr* 104:1072-1074, 1979.
29. Botsch H, Glatz J, Schulz E, and Wenzel K-W. Long-term follow-up using serial serum thyroglobulin determinations in patients with differentiated thyroid carcinoma. *Cancer* 52:1856-1859, 1983.
30. Bradbury SL, and Jakoby WB. Glycerol as an enzyme-stabilizing agent: Effects on aldehyde dehydrogenase. *Proc Natl Acad Sci (USA)* 69:2373-2376, 1972.
31. Bussolati G, and Monga G. Medullary carcinoma of the thyroid with atypical patterns. *Cancer* 44:1769-1777, 1979.
32. Böcker W, Dralle H, Hüsselmann H, Bay V, and Brassow M. Immunohistochemical analysis of thyroglobulin synthesis in thyroid carcinomas. *Virchows Arch (A)* 385:187-200, 1980.
33. Castagnoli A, Cappelli G, D'Agata A, Forni S, Milani S, and Pupi A. Can thyroglobulin assay really supplant radioiodine scans in patients with differentiated thyroid cancer? *Lancet* i:573-574, 1982.
34. Cavalieri RR, Searle GL, and Rosenberg LL. Studies of thyroglobulin of hypophysectomized rats. II. Nature of thyroidal iodoprotein after hypophysectomy of rats previously depleted of colloid by propylthiouracil. *Endocrinology* 86:10-17, 1970.
35. Charles MA, Dodson LE, Jr, Waldeck N, Hofeldt F, Ghaed N, Telepak R, Ownbey J, and Burstein P. Serum thyroglobulin levels predict total body iodine scan findings in patients with treated well-differentiated thyroid carcinoma. *Am J Med* 69:401-407, 1980.
36. Charles MA. Comparison of serum thyroglobulin with iodine scans in thyroid cancer. *J Endocrinol Invest* 5:267-271, 1982.
37. Colacchio TA, LoGerfo P, Colacchio DA, and Feind C. Radioiodine total body scan versus serum thyroglobulin levels in follow-up of patients with thyroid cancer. *Surgery* 91:42-45, 1982.

38. Cortese F, Schneider AB, and Salvatore G. Isopycnic centrifugation of thyroid iodoproteins: Selectivity of endocytosis. *Eur J Biochem* 68:121-129, 1976.
39. Cramarossa L, Andreoli M, Schneider A, and Edelhoach H. Effect of freezing in methylmercaptoimidazole on the structure of thyroglobulin. *Endocrinology* 89:741-748, 1971.
40. Daniel PM, Pratt OE, Roitt IM, and Torrigiani G. The release of thyroglobulin from the thyroid gland into thyroid lymphatics; the identification of thyroglobulin in the thyroid lymph and in the blood of monkeys by physical and immunological methods and its estimation by radioimmunoassay. *Immunology* 12:489-504, 1967.
41. Daniel PM, Pratt OE, Roitt IM, and Torrigiani G. Thyroglobulin in the lymph draining from the thyroid gland and in the peripheral blood of rats. *Q J Exp Physiol* 52:184-199, 1967.
42. Date J, Feldt-Rasmussen U, Hyltoft Petersen P, and Bech K. An improved co-precipitation assay for determination of thyroglobulin antibodies. *Scand J Clin Lab Invest* 40:37-44, 1980.
43. Dimitriu V, Zamfir-Grigorescu D, Aman E, Oprescu M, and Simionescu L. A radioimmunoassay system for human thyroglobulin. *Endocrinologie* 19:235-241, 1981.
44. Eber M, Abecassis J, Grob JC, Ott G, and Methlin G. Immunoradiometric assay for human thyroglobulin and variations in thyroid pathology. *Clin Chim Acta* 105:51-58, 1980.
45. Echenique RL, Kasi L, Haynie TP, Glenn HJ, Samaan NA, and Stratton Hill C. Critical evaluation of serum thyroglobulin levels and I-131 scans in post-therapy patients with differentiated thyroid carcinoma: Concise communication. *J Nucl Med* 23: 235-240, 1982.
46. Edelhoach H. The structure of thyroglobulin and its role in iodination. *Recent Progr Horm Res* 21:1-31, 1965.
47. Eggo MC, Burrow GN, Alexander NM, and Gordon JH. Iodination and the structure of human thyroglobulin. *J Clin Endocrinol Metab* 51:7-11, 1980.
48. Ekholm R, and Strandberg U. Studies on the protein synthesis in the thyroid. III. In vivo incorporation of leucine-³H into thyroglobulin of microsomal subfractions of the rat thyroid. *J Ultrastruct Res* 22:252-273, 1968.
49. Endo Y, Nakano J, Ohtaki S, Izumi M, Hamaguchi Y, Yoshitake S, and Ishikawa E. An enzyme immunoassay for the measurement of thyroglobulin in human serum. *Clin Chim Acta* 95:325-336, 1979.
50. Ewins DL, and Wilkin TJ. A clinical comparison of the enzyme-linked immunosorbent assay (ELISA) and haemagglutination (TRC)

in the routine detection of antithyroglobulin antibodies. *Acta Endocrinol (Copenh)* 103:216-222, 1983.

51. Feldt-Rasmussen U, Bech K, Nielsen H, and Date J. Circulating thyroglobulin, anti-thyroglobulin and immune complexes in patients with thyroid disease. *Ann Endocrinol (Paris)* 38:51 A, 1977.
52. Feldt-Rasmussen U, Petersen PH, Nielsen H, Date J, and Madsen CM. Thyroglobulin of varying molecular sizes with different disappearance rates in plasma following subtotal thyroidectomy. *Clin Endocrinol* 9:205-214, 1978.
53. Feldt-Rasmussen U, Bech K, and Date J. Serum thyroglobulin in patients with toxic and non-toxic goitres compared to sex- and aged-matched control subjects. *Acta Endocrinol (Copenh)* 91:264-270, 1979.
54. Feldt-Rasmussen U, Bech K, Date J, Hyltoft Petersen P, and Johansen K. A prospective study on the differential changes in serum thyroglobulin and its autoantibodies during propylthiouracil or radioiodine therapy of patients with Graves' disease. *Acta Endocrinol (Copenh)* 99:379-385, 1982.
55. Feldt-Rasmussen U, Holten I, and Sand Hansen H. Influence of thyroid substitution therapy and thyroid autoantibodies on the value of serum thyroglobulin in recurring thyroid cancer. *Cancer* 51:2240-2244, 1983.
56. Feldt-Rasmussen U, Perrild H, Bech K, Bliddal H, Date J, Høier Madsen M, Nordfang O, Ryder LP, Thomsen M, Kappelgaard E, and Nielsen H. Discrepancy between haemagglutination and radioimmunological techniques for measurement of serum thyroglobulin autoantibodies. *Allergy* 38:49-56, 1983.
57. Frohman LA, Baron MA, and Schneider AB. Plasma immunoreactive TSH: Spurious elevation due to antibodies to bovine TSH which cross-react with human TSH. *Metabolism* 31:834-840, 1982.
58. Galligan JP, Winship J, van Doorn T, and Mortimer RH. A comparison of serum thyroglobulin measurements and whole body ^{131}I scanning in the management of treated differentiated thyroid carcinoma. *Aust NZ J Med* 12:248-254, 1982.
59. Gardner DF, Rothman J, and Utiger RD. Serum thyroglobulin in normal subjects and patients with hyperthyroidism due to Graves' disease: Effects of T_3 iodide, ^{131}I and antithyroid drugs. *Clin Endocrinol* 11:585-594, 1979.
60. Grabar P. Hypothesis. Auto-antibodies and immunological theories: An analytical review. *Clin Immunol Immunopathol* 4:453-466, 1975.
61. Gärtner R, Hainzinger A, Horn K, and Pickardt RC. Evidence for autonomous thyroglobulin release from euthyroid and hyperthy-

roid nodular goiter - Thyroglobulin, a possible helpful parameter in diagnosis of non-malignant thyroid disorders. *Klin Wochenschr* 61:737-741, 1983.

62. Hackett E, Beech M, and Forbes IJ. Thyroglobulin antibodies in patients without clinical disease of the thyroid gland. *Lancet* ii:402-404, 1960.
63. Hagemann J, and Schneider C. Control of treatment of differentiated thyroid carcinoma by measurement of thyroglobulin in serum. IAEA. International symposium on radioimmunoassay and related procedures in medicine. West Berlin. 1977 p 363-368.
64. Hales M, Rosenau W, Okerlund MD, and Galante M., Carcinoma of the thyroid with a mixed medullary and follicular pattern. Morphologic, immunohistochemical, and clinical laboratory studies. *Cancer* 50:1352-1359, 1982.
65. Hedinger C, and Sobin LH. Histological typing of thyroid tumours. International histological classification of tumours. WHO 1974 No 11. p. 16-26. Geneva.
66. Heimann P, Ljunggren J-G, Löwhagen T, and Hjern B. Oxyphilic adenoma of the human thyroid. A morphological and biochemical study. *Cancer* 31:246-254, 1973.
67. Hill OW. Thyroglobulin antibodies in 1297 patients without thyroid disease. *Br Med J* I:1793-1796, 1961.
68. Hjort T. Determination of serum-thyroglobulin by a haemagglutination-inhibition test. *Lancet* i:1262-1264, 1961.
69. Hüfner M, Pollmann H, Grussendorf M, und Schenk P. Die Bedeutung der Thyreoglobulinbestimmung im Serum bei der Nachsorge von Patienten mit differenziertem Schilddrüsenkarzinom. *Schweiz Med Wochenschr* 110:159-162, 1980.
70. Ikekubo K, Jutton J, and Schneider AB. Radioimmunoassay of human thyroglobulin with use of "thyroglobulin-free" plasma prepared by ultracentrifugation as diluent. *Clin Chem* 26:1566-1568, 1980.
71. Ikekubo K, Pervos R, and Schneider AB. Clearance of normal and tumor-related thyroglobulin from the circulation of rats: Role of the terminal sialic acid residues. *Metabolism* 29:673-681, 1980.
72. Ikekubo K, Kishihara M, Sanders J, Jutton J, and Schneider AB. Differences between circulating and tissue thyroglobulin in rats. *Endocrinology* 109:427-432, 1981.
73. Inoue K, and Taurog A. Freezing-induced alterations in thyroglobulin from iodine deficient and iodine sufficient rats. *Endocrinology* 83:833-844, 1968.

74. Jeevanram RK, Shah DH, Sharma SM, and Ganatra RD. Disappearance rate of endogenously radioiodinated thyroglobulin and thyroxine after radioiodine treatment. *Cancer* 49:2281-2284, 1982.
75. Jänsch A, Heinze HG, und Hast B. Serum-Thyreoglobulin (S-hTG): Ein Tumormarker bei Patienten mit differenziertem Schilddrüsenkarzinom. *Strahlentherapie* 157:381-392, 1981.
76. Kallifronas MD, Ioannou D, Michopoulos I, Yatromanolakis N, Kipiotis N, and Batrinos ML. Incidence of thyroglobulin and microsomal antibodies in 573 healthy young males. *Acta Endocrinol (Copenh) Suppl* 244:61, 1981.
77. Kawamura S, Kishino B, Tajima K, Mashita K and Tarui S. Serum thyroglobulin changes in patients with Graves' disease treated with long term antithyroid drug therapy. *J Clin Endocrinol Metab* 56:507-512, 1983.
78. Lamas L, and Ingbar SH. The effect of varying iodine content on the susceptibility of thyroglobulin to hydrolysis by thyroid acid protease. *Endocrinology* 102:188-197, 1978.
79. Lamas L, and Santisteban P. Re-evaluation of some factors affecting the stability of rat thyroglobulin. *Biochim Biophys Acta* 621:265-272, 1980.
80. Leibo SP, and Jones RF. Freezing of the chromoprotein phycoerythrin from the red alga *Porphyridium cruentum*. *Arch Biochem Biophys* 106:78-88, 1964.
81. Lerner RA. Tapping the immunological repertoire to produce antibodies of predetermined specificity. *Nature* 299:592-596, 1982.
82. Ljunggren J-G, Löwhagen T, and Hjern B. The formation of thyroglobulin in human thyroid medullary carcinoma. *Acta Endocrinol (Copenh)* 74:105-110, 1973.
83. Lo Gerfo P, Colacchio D, Stillman T, and Feind C. Serum thyroglobulin and recurrent thyroid cancer. *Lancet* i:881-882, 1977.
84. Lo Gerfo P, Colacchio T, Colacchio D, and Feind C. Serum clearance rates of immunologically reactive thyroglobulin. *Cancer* 42:164-166, 1978.
85. Lo Gerfo P, Colacchio TA, Colacchio DA, and Feind CR. Effects of TSH stimulation on serum thyroglobulin in metastatic thyroid cancer. *J Surg Oncol* 14:195-200, 1980.
86. Maloof F, Sato G, and Soodak M. Dissociation of iodination from protein synthesis in the rat thyroid. *Medicine (Baltimore)* 43:375-378, 1964.
87. McDougall IR, and Bayer MF. Follow-up of patients with differentiated thyroid cancer using serum thyroglobulin measured

- by an immunoradiometric assay. Comparison with I-131 total body scans. *J Nucl Med* 21:741-744, 1980.
88. Metzger H, Sharp GC, and Edelhoach H. The properties of thyroglobulin VII. The immunologic activity of thyroglobulin fragments. *Biochemistry* 1:205-214, 1962.
 89. Mori T, and Kriss JP. Measurements by competitive binding radioassay of serum anti-microsomal and anti-thyroglobulin antibodies in Graves' disease and other thyroid disorders. *J Clin Endocrinol Metab* 33:688-698, 1971.
 90. Müller-Brand J, Fridrich R, Spicher E, und Staub JJ. Thyreoglobulin als Tumormarker und Thalliumszintigraphie zur Verlaufskontrolle beim differenzierten Schilddrüsenkarzinom. *Schweiz Med Wochenschr* 113:325-327, 1983.
 91. Nadler NJ, Young BA, Leblond CP, and Mitmaker B. Elaboration of thyroglobulin in the thyroid follicle. *Endocrinology* 74:333-354, 1964.
 92. Nève P, Chailly P, Van Haelst L, Mahieux A, et Bastenie PA. Contribution a l'étude de la thyroïdite lymphocytaire atrophique: état de pré-myxoedème. *Rev Franc Etudes Clin et Biol* 11: 710-713, 1966.
 93. Ochi Y, Hachiya T, Yoshimura M, Miyazaki T, Majima T, Kaimatsu I, and Takahashi H. Radioimmunoassay for estimation of thyroglobulin in human serum. *Endocrinol Jpn* 22:351-356, 1975.
 94. Oswald Ad. Die Eiweisskörper der Schilddrüse. *Hoppe-Seyler's Z Physiol Chem* 27:14-49, 1899.
 95. Owen CA, Jr. A review of "auto-immunization" in Hashimoto's disease. *J Clin Endocrinol Metab* 18:1015-1023, 1958.
 96. Owen SG, and Smart GA. Thyroid antibodies in myxoedema. *Lancet* ii:1034-1036, 1958.
 97. Pacini F, Pinchera A, Giani C, Grasso L, Doveri F, and Baschieri L. Serum thyroglobulin in thyroid carcinoma and other thyroid disorders. *J Endocrinol Invest* 3:283-292, 1980.
 98. Pacini F, Pinchera A, Giani C, Grasso L, and Baschieri L. Serum thyroglobulin concentrations and ¹³¹I whole body scans in the diagnosis of metastases from differentiated thyroid carcinoma (after thyroidectomy). *Clin Endocrinol (Oxf)* 13:107-110, 1980.
 99. Peake RL, Willis DB, Asimakis GK, Jr, and Deiss WP, Jr. Radio-immunologic assay for antithyroglobulin antibodies. *J Lab Clin Med* 84:907-919, 1974.
 100. Persson PS. Cytodiagnosis of thyroiditis. A comparative study of cytological, histological, immunological and clinical find-

ings in thyroiditis, particularly in diffuse lymphoid thyroiditis. *Acta Med Scand Suppl.* 483, 1968.

101. Pezzino V, Cozzani P, Filetti S, Galbiati A, Lisi E, Squatrito S, and Vigneri R. A radioimmunoassay for human thyroglobulin: methodology and clinical applications. *Eur J Clin Invest* 7:503-508, 1977.
102. Pinchera A, Mariotti S, Vittì P, Tosi M, Grasso L, Pacini F, Buti R, and Baschieri L. Interference of serum thyroglobulin in the radioassay for serum antithyroglobulin antibodies *J Clin Endocrinol Metab* 45:1077-1088, 1977.
103. Pitt-Rivers R. Proteolytic fragments of thyroglobulin. In *Molecular mechanisms and regulation of thyroid hormone biosynthesis*. Simposio int, 1972, Roma 1975.
104. Pohl RG. Solute redistribution by recrystallization. *J Appl Physics* 25:1170-1178, 1954.
105. Refetoff S, and Lever EG. The value of serum thyroglobulin measurement in clinical practice. *JAMA* 250:2352-2357, 1983.
106. Roitt IM, Doniach D, Campbell PN, and Hudson RV. Auto-antibodies in Hashimoto's disease (lymphadenoid goitre). *Lancet* ii:820-821, 1956.
107. Roitt IM, and Doniach D. Human auto-immune thyroiditis: serological studies. *Lancet* ii:1027-1033, 1958.
108. Roitt IM, and Torrigiani G. Identification and estimation of undegraded thyroglobulin in human serum. *Endocrinology* 81:421-429, 1967.
109. Rolland M, and Lissitzky S. Endogenous proteolytic activity and constituent polypeptide chains of sheep and pig 19S thyroglobulin. *Biochim Biophys Acta* 427:696-707, 1976.
110. Sain A, Sham R, Singh A, and Silver L. Erroneous thyroid-stimulating hormone radioimmunoassay results due to interfering antiovine thyroid-stimulating hormone antibodies. *Am J Clin Pathol* 71:540-542, 1979.
111. Salabè GB, Salabè H, Dominici R, Davoli C, and Andreoli M. Radioimmunoassay for human antithyroglobulin antibodies. II. Determination of antigen-binding capacity. *J Clin Endocrinol Metab* 39:1125-1132, 1974.
112. Salvatore G, and Edelhoch H. Chemistry and biosynthesis of thyroid iodoproteins. In: *Hormonal proteins and peptides*, vol I. Ed CH Li. Acad Press, New York, 1973, p 201.
113. Schatz H, Grebe S, Mäser E, Teuber J, Horn W, Schröder O, and Schatz Ch. Serum-Thyreoglobulinspiegel als Tumormarker bei Schilddrüsencarcinom. *Klin Wochenschr* 60:457-464, 1982.

114. Schlumberger M, Fragu P, Vignal A et Tubiana M. Dosage de la thyroglobuline humaine dans le sérum et le tissu thyroïdien. *Ann Endocrinol (Paris)* 40:439-440, 1979.
115. Schlumberger M, Charbord P, Fragu P, Lumbroso J, Parmentier C, and Tubiana M. Circulating thyroglobulin and thyroid hormones in patients with metastases of differentiated thyroid carcinoma: relationship to serum thyrotropin levels. *J Clin Endocrinol Metab* 51:513-519, 1980.
116. Schlumberger M, Fragu P, Parmentier C and Tubiana M. Thyroglobulin assay in the follow-up of patients with differentiated thyroid carcinomas: comparison of its value in patients with or without normal residual tissue. *Acta Endocrinol (Copenh)* 98: 215-221, 1981.
117. Schlumberger M, Fragu P, Travagli JP, Gardet P, Lumbroso J, Charbord P, Lacour J, et Parmentier C. Intérêt du dosage de la thyroglobuline sérique dans le cancer de la thyroïde. *Nouv Presse Med* 11:3101-3105, 1982.
118. Schneider AB, and Edelhoch H. Equilibrium density centrifugation of thyroglobulin in RbCl. Effect of iodine. *J Biol Chem* 246:6592-6598, 1971.
119. Schneider AB, Favus MJ, Stachura ME, Arnold JE, Ryo UY, Pinsky S, Colman M, Arnold MJ, and Frohman LA. Plasma thyroglobulin in detecting thyroid carcinoma after childhood head and neck irradiation. *Ann Intern Med* 86:29-34, 1977.
120. Schneider AB, and Pervos R. Radioimmunoassay of human thyroglobulin: Effect of antithyroglobulin autoantibodies. *J Clin Endocrinol Metab* 47:126-137, 1978.
121. Schneider AB, Ikekubo K, and Jutton J. Characterization and metabolism of circulating thyroglobulin in thyroid cancer. In: *Advances in thyroid neoplasia* p. 202-211. Eds. Andreoli M, Monaco F, and Robbins J. Field Educational, Italia, 1981.
122. Schneider AB, Line BR, Goldman JM, and Robbins J. Sequential serum thyroglobulin determinations, ^{131}I scans, and ^{131}I uptakes after triiodothyronine withdrawal in patients with thyroid cancer. *J Clin Endocrinol Metab* 53:1199-1206, 1981.
123. Schneider AB, Ikekubo K, and Kuma K. Iodine content of serum thyroglobulin in normal individuals and patients with thyroid tumors. *J Clin Endocrinol Metab* 57:1251-1256, 1983.
124. Seed RW, and Goldberg IH. Biosynthesis of thyroglobulin: Relationship to RNA-template and precursor protein. *Proc Natl Acad Sci (USA)* 50:275-282, 1963.
125. Sela M, Schechter B, Schechter I, and Borek F. Antibodies to sequential and conformational determinants. *Cold Spring Harbor Symp Quant Biol* 32:537-545, 1967.

126. Shah D, Dandekar S, and Ganatra RD. Thyroglobulin levels in serum and saliva of patients with differentiated thyroid carcinoma. *Proc Indian Acad Sci* 87B:169-175, 1978.
127. Shah DH, Dandekar SR, Jeevanram RK, Kumar A, Sharma SM, and Ganatra RD. Serum thyroglobulin in differentiated thyroid carcinoma: histological and metastatic classification. *Acta Endocrinol (Copenh)* 98:222-226, 1981.
128. Shifrin S, and Parrott CL. Influence of glycerol and other polyhydric alcohols on the quaternary structure of an oligomeric protein. *Arch Biochem Biophys* 166:426-432, 1975.
129. Shlossberg AH, Jacobson JC, and Ibbertson HK. Serum thyroglobulin in the diagnosis and management of thyroid carcinoma. *Clin Endocrinol (Oxf)* 10:17-27, 1979.
130. Shulman S, Rose NR, and Witebsky E. Studies on organ specificity III. Ultracentrifugal and electrophoretic examinations of thyroid extracts. *J Immunol* 75:291-300, 1955.
131. Simon C, Roques M, Torresani J, and Lissitzky S. Effect of propylthiouracil on the iodination and maturation of rat thyroglobulin. *Acta Endocrinol (Copenh)* 53:271-285, 1966.
132. Sinadinović J, Krainčanić M, Marinković B, Jovanović M, Petrović N, and Liewendahl K. Susceptibility to proteolysis of thyroglobulin from rats and guinea-pigs treated with excess iodide. *Acta Endocrinol (Copenh)* 99:232-238, 1982.
133. Spiro MJ. Studies on the protein portion of thyroglobulin. Amino acid compositions and terminal amino acids of several thyroglobulins. *J Biol Chem* 245:5820-5826, 1970.
134. Spiro MJ. Subunit heterogeneity of thyroglobulin. *J Biol Chem* 248:4446-4460, 1973.
135. Spiro RG, and Spiro MJ. The carbohydrate composition of the thyroglobulins from several species. *J Biol Chem* 240:997-1001, 1965.
136. Taborsky G. Effect of freezing and thawing on the conformation of phosvitin. *J Biol Chem* 245:1054-1062, 1970.
137. Taborsky G. Solute redistribution in some multicomponent aqueous systems on freezing. *J Biol Chem* 245:1063-1068, 1970.
138. Tang Fui SCNG, Hoffenberg R, Maisey MN, and Black EG. Serum thyroglobulin concentrations and whole-body radioiodine scan in follow-up of differentiated thyroid cancer after thyroid ablation. *Br Med J* II:298-300, 1979.
139. Tatumi K, Suzuki Y, and Sinohara H. Clearance of circulating desialylated thyroglobulins in the rat. *Biochim Biophys Acta* 583:504-511, 1979.

140. Tishler PV, and Ingbar SH. Correlative effects of puromycin on ¹³¹I metabolism and amino acid incorporation by calf thyroid slices. *Endocrinology* 76:295-300, 1965.
141. Torrigiani G, Doniach D, and Roitt IM. Serum thyroglobulin levels in healthy subjects and in patients with thyroid disease. *J Clin Endocrinol Metab* 29:305-314, 1969.
142. Uller RP, van Herle AJ, and Chopra IJ. Comparison of alterations in circulating thyroglobulin, triiodothyronine and thyroxine in response to exogenous (bovine) and endogenous (human) thyrotropin. *J Clin Endocrinol Metab* 37:741-745, 1973.
143. Uller RP, and van Herle AJ. Effect of therapy on serum thyroglobulin levels in patients with Graves' disease. *J Clin Endocrinol Metab* 46:747-755, 1978.
144. Unger J, van Heuverswyn B, Decoster C, Cantraine F, Mockel J, and van Herle A. Thyroglobulin and thyroid hormone release after intravenous administration of bovine thyrotropin in man. *J Clin Endocrinol Metab* 51:590-594, 1980.
145. Valenta L, and Lissitzky S. The importance of the ionic strength of sucrose gradients in the determination of the sedimentation coefficient of poorly iodinated or newly synthesized thyroglobulin. *Biochim Biophys Acta* 236:376-383, 1971.
146. Valenta LJ, Michel-Bechet M, Mattson JC, and Singer FR. Microfollicular thyroid carcinoma with amyloid rich stroma, resembling the medullary carcinoma of the thyroid (MCT). *Cancer* 39:1573-1586, 1977.
147. van der Walt BJ, and van Jaarsveld PP. Isolation procedures for thyroglobulin: Effects of phenylmetanesulfonyl fluoride and freezing. *S Afr J Med Sci* 41:197-206, 1976.
148. van Herle AJ, Uller RP, Matthews NL, and Brown J. Radioimmunoassay for measurement of thyroglobulin in human serum. *J Clin Invest* 52:1320-1327, 1973.
149. van Herle AJ, Klandorf H, and Uller RP. A radioimmunoassay for serum rat thyroglobulin. *Physiologic and pharmacological studies.* *J Clin Invest* 56:1073-1081, 1975.
150. van Herle AJ, and Uller RP. Elevated serum thyroglobulin. A marker of metastases in differentiated thyroid carcinomas. *J Clin Invest* 56:272-277, 1975.
151. van Herle AJ, Vassart G, and Dumont JE. Control of thyroglobulin synthesis and secretion (First of two parts). *N Engl J Med* 301:239-249, 1979.
152. van Herle AJ, Vassart G, and Dumont JE. Control of thyroglobulin synthesis and secretion (Second of two parts). *N Engl J Med* 301:307-314, 1979.

153. Vassart G, Refetoff S, Brocas H, Dinsart C, and Dumont JE. Translation of thyroglobulin 33S messenger RNA as a means of determining thyroglobulin quaternary structure. *Proc Natl Acad Sci (USA)* 72:3839-3843, 1975.
154. Whittingham S, Irwin J, Mackay IR, Marsh S, and Cowling DC. Autoantibodies in healthy subjects. *Aust Ann Med* 18:130-134, 1969.
155. Williams ED, and Doniach I. The post-mortem incidence of focal thyroiditis. *J Path Bact* 83:255-264, 1962.
156. Yoshida H, Amino N, Yagawa K, Uemura K, Satoh M, Miyai K, and Kumahara Y. Association of serum antithyroid antibodies with lymphocytic infiltration of the thyroid gland: Studies of seventy autopsied cases. *J Clin Endocrinol Metab* 46:859-862, 1978.
157. Zeytinoglu FN, Gagel RF, Tashjian AH, Jr, Hammer RA, and Leeman SE. Characterization of neurotensin production by a line of rat medullary thyroid carcinoma cells. *Proc Natl Acad Sci (USA)* 77:3741-3745, 1980.

Purification and storage of thyroglobulin. Two important factors influencing the radioimmunoassay for thyroglobulin

U.-B. ERICSSON, I. LARSSON & J. I. THORELL

Department of Internal Medicine and Section for Nuclear Medicine at the Department of Clinical Chemistry, University of Lund, Malmö General Hospital, Malmö, Sweden

Ericsson, U.-B., Larsson, I. & Thorell, J.I. Purification and storage of thyroglobulin. Two important factors influencing the radioimmunoassay for thyroglobulin. *Scand. J. clin. Lab. Invest.* 44, 000-000, 1984.

The effect upon the assay of the quality of the thyroglobulin (Tg) used as standard and tracer was evaluated by comparison of two preparations, one purified with protease inhibitors added (Tg-PI) and the other without (Tg-O). Tg-PI proved more stable than Tg-O. After freezing in phosphate buffered saline (PBS) almost all ^{125}I -Tg-O was found to have dissociated into 12 S Tg, while only about half the ^{125}I -Tg-PI had done so. Storage in glycerol, 500 g/l, at -20°C or freezing in goat serum improved the quality of the ^{125}I -Tg markedly, but Tg-PI still remained more stable than Tg-O.

In addition, the two antisera tested gave different results in the RIA with Tg-PI and Tg-O. With one antiserum a gradual loss of Tg immunoreactivity occurred parallel to the dissociation of Tg, while no such effect was noted with the other antiserum. This difference is believed to depend on varying proportions of conformational antibodies in the antisera, the binding sites for the conformational antibodies being distorted by the dissociation of the Tg molecule, while the binding sites for the sequential antibodies remain intact.

Key-words: thyroglobulin; thyroglobulin radioimmunoassay; purification methods; protease inhibitors

Ulla-Britt Ericsson, Department of Internal Medicine, Malmö General Hospital, S-214 01 Malmö, Sweden

Though numerous reports on the technical applications of the radioimmunoassay for thyroglobulin (Tg-RIA) have appeared in recent years [3, 6, 10, 18, 23], less attention has been devoted to the technical problems arising in its performance, other than that of the interference of thyroglobulin antibodies in the assay [2,

11, 15, 20, 24, 25]. The heterogeneity and instability of the Tg molecule is well established [1, 13, 21-29, 30], but the influence of these factors on the Tg-RIA has not been evaluated to any extent despite the wide variation of the normal serum concentrations reported from different laboratories

Several methodological problems, stemming from the properties of the Tg, arose during the development of the Tg-RIA. The effect upon the assay of the quality of the Tg used as standard and tracer was evaluated. We report here the development of an improved method of purifying Tg and its application in a radioimmunoassay modified to suit the special characteristics of Tg.

MATERIALS AND METHODS

Purification of thyroglobulin

Human thyroid tissue was obtained from patients with multinodular goitre undergoing subtotal thyroidectomy. After removal, glands were immediately frozen in liquid nitrogen and stored at -70°C until needed for tissue extraction. The purification procedure was performed both with and without the addition of protease inhibitors to the buffers. Thyroglobulin purified in the presence of protease inhibitors (PI) was called Tg-PI and Tg purified without the protection of PI was called Tg-O.

Tissue extraction. Five grams of thyroid tissue were homogenized for 2 min with a Polytron PTA 10-35 (Kinematica, Lucerne, Switzerland) in the presence of a mixture of protease inhibitors (PI) in NaCl, 0.15 mol/l containing sodium phosphate, 0.0035 mol/l and 0.02% NaN_3 , pH 7.0 (PBS), 2 ml/g tissue. The protease inhibitors used were: Leupeptin (Peninsula, USA) 0.1 mmol/l, pepstatin (Sigma Chemical Co., USA) 0.1 mmol/l, PMSF (phenyl-methyl-sulphonyl fluoride, Sigma Chemical Co., USA) 0.5 mol/l in methanol (1 mmol/l), benzamidine (Sigma Chemical Co., USA) 1 mmol/l, TPCK (l-tosylamide-2-phenylchloro-methyl ketone, Sigma Chemical Co., USA) 0.1 mmol/l, soybean trypsin inhibitor (approx. 10,000 BAEE units/mg protein), 50 $\mu\text{g}/\text{ml}$, DFP (diisopropyl-fluoro phosphate, Fluka, FRG) 1 mmol/l and trasylol (10,000 KIU/ml, Bayer, FRG) 100 $\mu\text{g}/\text{ml}$. The suspension was stirred for 2 h at $+4^{\circ}\text{C}$, filtered through gauze and centrifuged for 10 min at $+4^{\circ}\text{C}$ and 10,000 g. The supernatant was further purified with gel chromatography.

In a control experiment Tg-O was extracted by an identical procedure but without the presence of protease inhibitors.

Gel chromatography When not otherwise

stated the purification procedure was performed at $+4^{\circ}\text{C}$. Eight millilitres of the supernatant were applied to a 2.6×60 cm column of Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with PBS containing benzamidine, 1 mmol/l. Tg-O was eluted with the same buffer but without benzamidine. Fractions of 3 ml were collected and the absorbance at 280 nm was recorded on a 'Uvicord' (LKB, Sweden). The presence of Tg in the fractions was determined by fused rocket immunoelectrophoresis [31] against anti-whole-human serum (100 SE, DAKO, Denmark) with anti-Tg in the intermediary gel as described by Feldt-Rasmussen, 1975 [8]. Fractions corresponding to the void volume, shown to contain a high amount of Tg, were pooled and concentrated approximately 10 $\times$ on a Minicon (Amicon Corp., Mass., USA, filter cut off 100,000 Daltons). The concentrated solution was again chromatographed on a 1.6×100 cm column of Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) and eluted with PBS containing benzamidine, 1 mmol/l. Tg-O was eluted without benzamidine in the elution buffer. Fractions of 1.7 ml were collected and the absorbance at 280 nm was recorded. The presence of Tg in the fractions was determined by fused rocket electrophoresis. The fractions shown to contain the highest amounts of Tg were pooled and concentrated as described above. The protein concentration in the solution was determined according to Lowry [14]. The Tg was stored in PBS containing 50% glycerol (Merck, FRG). The Tg used as standard in the RIA was prepared from the glycerol solution by lyophilization and weighing.

Iodination of thyroglobulin

Tg was iodinated with Na^{125}I (Code IMS 30, Amersham International, Amersham, England), using the lactoperoxidase (LPO) method [34]. To remove NaN_3 , Tg was desalinated on a PD-10 column (Pharmacia Fine Chemicals, Uppsala, Sweden) before iodination. Typically, 10 μg Tg in 50 μl phosphate buffer (0.05 mol/l, pH 7.5) was incubated with 500 μCi (18.5 MBq) ^{125}I in 5 μl NaOH (pH 7–11), 4 μg LPO in 2 μl phosphate buffer and 60 ng H_2O_2 in 2 μl H_2O (0.88 mmol/l) for 10 sec. When Tg was stored in 50% glycerol 150 ng H_2O_2 was used and the reaction time was prolonged to 30–60 sec. The

incubate was then diluted with 0.5 ml phosphate buffer, 0.05 mol/l, pH 7.5, desalinated on a 1×14 cm column of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), pre-treated with 1 ml 2% bovine serum albumin in a barbital buffer, 0.075 mol/l, pH 8.5. Fractions of 1 ml were collected into 1 ml BBSA and stored in 50% glycerol at -20°C.

One millilitre of the ^{125}I -Tg was chromatographed on a 2.6×100 cm column of Ultrogel ACA 22 (LKB, Sweden) and eluted with BBSA. Fractions of 3.7 ml were collected and their immunoreactivity tested in a Tg-RIA. The molecular size distributions of eluates from the chromatography fractions of the iodinated Tg were analysed by electrophoresis in a polyacrylamide gradient gel PAA 4/30 (Pharmacia Fine Chemicals, Uppsala, Sweden) in a Tris buffer, 0.09 mol/l containing borate, 0.08 mol/l and Na_2EDTA , 0.003 mol/l, pH 8.35. The following marker proteins were used: α_2 -macroglobulin (820,000 daltons), apoferritin (443,000 daltons), catalase (240,000 daltons), ceruloplasmin (132,000 daltons) and albumin (67,000 daltons).

Thyroglobulin radioimmunoassay

Preparation of antiserum. Tg-PI and a preparation purified by Dr van Herle (Tg-vH) were used to raise the first antibody in the rabbit. The antisera were produced as follows (numbers in parentheses denote dosages of Tg-vH): 50 µg (1.0 mg) human Tg, dissolved in 1.0 ml 0.9% NaCl and emulsified in 1.0 ml of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich., USA), was given to each rabbit by the multiple site, intradermal technique [36]. Booster injections were given twice at various intervals, with a dosage of 100 µg (1.0 mg) and 200 µg (1.0 mg), respectively. Starting 2 weeks after the last booster injection, antiserum was collected weekly and stored at -20°C. The antisera against Tg-PI (k-8102) and against Tg-vH (k-7502) were used at dilutions of 1:20,000 and 1:250,000, respectively.

Radioimmunoassay procedure. A slight modification of the double antibody RIA described by van Herle *et al.* [10] was used. The stock standard was stored at -20°C either in 0.075 M barbital buffer, pH 8.6, containing 0.25% bovine serum albumin (Armour, Eastbourne, England) or in normal goat serum (NGS). Three different Tg preparations were used for

preparing the standards: Tg-PI, Tg-O and Tg-vH. Standards of 6.25, 12.5, 25, 50, 100, and 200 µg Tg per litre were prepared by diluting the stock standard with NGS. 100 µl of the standard solution or the serum sample was preincubated for 48 h at +4°C with 200 µl of antiserum. Two hundred microlitres ^{125}I -Tg (approx. 10,000 cpm) tracer solution were then added, and the tubes were incubated for another 72 h at +4°C. Finally 50 µl goat anti-rabbit serum (diluted 1:10 in barbital buffer containing 0.25% EDTA and 0.25% bovine serum albumin) was added as precipitating antibody along with 50 µl rabbit serum diluted 1:250. Following a further 24-h incubation at +4°C the tubes were centrifuged for 15 min at +4°C and 2400 g and the supernatants discarded. The precipitates were counted in an automatic gamma counter.

Cross reactivity was tested for T_3 , T_4 , MIT, and DIT. Amounts of up to 1000 µg/l did not interfere with the binding of ^{125}I -Tg.

Statistics

Analysis of variation with a two-way classification and paired *t*-test were used for statistical calculations [4].

RESULTS

Purification of thyroglobulin

The difference in the elution pattern of the thyroid extracts, purified both with and without the presence of PI, is shown in Fig. 1. In the presence of PI, Tg eluted as one dominating peak, with less than one-third of the proteins emerging as side fractions of lower or higher molecular size. In the absence of PI, the Tg peak was less well defined with more than 50% of the protein appearing in fractions of lower molecular size.

^{125}I -thyroglobulin

With radioiodination of Tg-PI the incorporation of ^{125}I into the thyroglobulin was 70–80% (specific activity 1.30–1.48 MBq/µg), while with Tg-O and Tg-vH the yields were only 20–50% (specific activity 0.37–0.93 MBq/µg).

Freshly iodinated Tg-PI and Tg-O showed similar elution patterns when separated on an Ultrogel ACA 22 column. The radioactivity eluted as one dominating peak containing

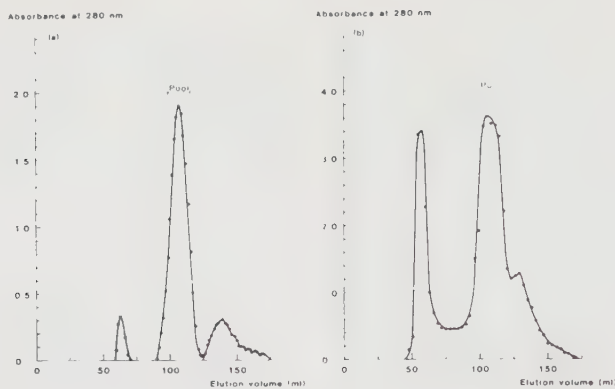


FIG. 1. Elution patterns from the final purification step on a Sepharose 4B column (1.6×100 cm) of the human Tg purified with the addition of protease inhibitors to the buffers (a) and without (b). Flow rates 12 ml/h (a) and 15 ml/h (b). Fraction volume 1.7 ml (a) and 3.0 ml (b). Fractions (indicated by the black bar), which were shown to contain Tg as determined by fused rocket electrophoresis, were pooled and used for further studies.

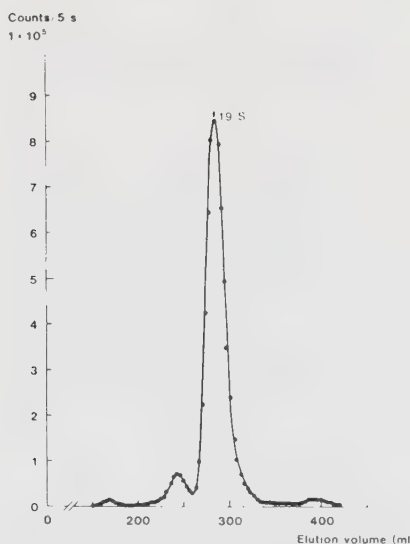


FIG. 2. Elution pattern from Ultrogel ACA 22 (2.6×100 cm) of freshly iodinated Tg-PI. Flow rate 12 ml/h. Fraction volume 3.5 ml. The column was eluted with a barbital buffer, 0.075 mol/l, pH 8.5, containing bovine serum albumin, 20 g/l. 19S denotes fractions containing the largest proportion of ^{125}I -Tg in the 19S form as determined by polyacrylamide gel electrophoresis.

approximately 80% of the radioactivity, corresponding to 19S Tg (660,000 daltons) when run on a polyacrylamide gel electrophoresis (PAA 4/30) (see Fig. 2).

Freezing-thawing once altered the Ultrogel ACA 22 pattern, the principal change being the appearance of a peak eluting after that of the original 19S protein. On analysis in polyacrylamide gel electrophoresis, this peak was shown to contain 12S protein (330,000 daltons). Almost 50% of the 19S protein of the ^{125}I -Tg-PI preparation showed this conversion, while with the ^{125}I -Tg-O almost all protein was found in the 12S fraction after freezing, with a barely recognizable 'shoulder' of 19S Tg remaining (Fig. 3).

The application of the various preparations of radioiodinated Tg in the RIA revealed distinct differences. In a typical example the maximal binding (B_0) was 67% with ^{125}I -Tg-PI and 54% with ^{125}I -Tg-O. Freezing affected the latter Tg-preparation in particular, causing a reduction of its binding to 23% while that of Tg-PI only dropped to 64%.

If freezing-thawing was circumvented by storage in 50% glycerol at -20°C these changes were minimal. The elution pattern of ^{125}I -Tg-PI, stored in 50% glycerol, was essentially identical to that of freshly iodinated Tg (Fig. 4), while that of ^{125}I -Tg-O showed broadened

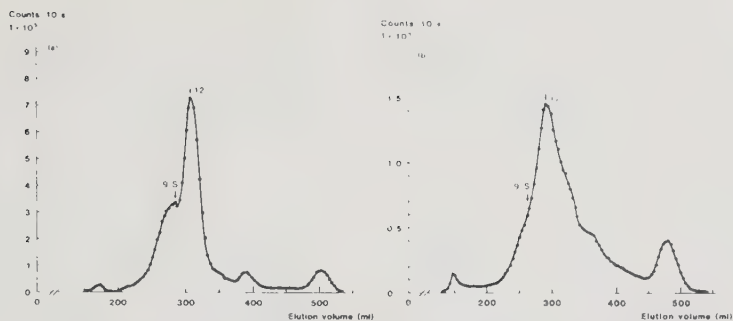


FIG. 3. Elution patterns from Ultrogel ACA 22 (2.6×100 cm) of ^{125}I -Tg after freezing in buffer at -20°C . The column was eluted with a barbital buffer, 0.075 mol/l, pH 8.5, containing bovine serum albumin, 20 g/L. (a) Tg purified with the addition of protease inhibitors (Tg-PI). Flow rate 12 ml/h. Fraction volume 3.7 ml. (b) Tg purified without the addition of protease inhibitors (Tg-O). Flow rate 15 ml/h. Fraction volume 3.7 ml. 19S and 12S denotes fractions containing the largest proportion of ^{125}I -Tg in the 19S and 12S forms as determined by polyacrylamide gel electrophoresis.

peaks and proportionally increased fractions of smaller size proteins. This was confirmed by analysis in polyacrylamide gel electrophoresis.

The storage of the ^{125}I -Tg in 50% glycerol at -20°C had very little influence on the binding. After one week the binding of ^{125}I -Tg-PI was 62%, and of ^{125}I -Tg-O 49%. Another effect of the storage of ^{125}I -Tg in glycerol was a reduction of the analytical error (Table 1).

Standards

When the Tg standard was prepared from a stock standard frozen in buffer, there was a progressive loss of immunoreactivity (Fig. 5). After one week there was already a significant change ($P < 0.01$), which increased gradually with the storage time. Read against this standard after 3 months, the apparent Tg concen-

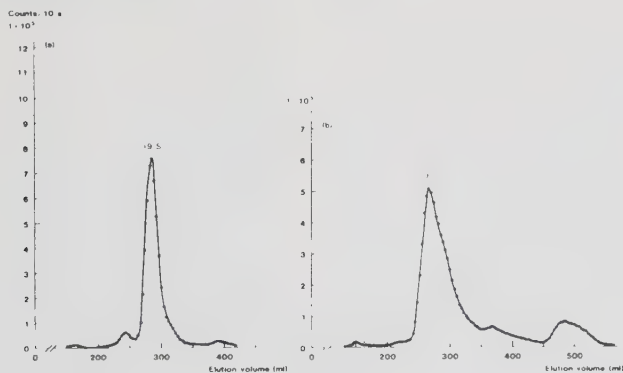


FIG. 4. Elution patterns from Ultrogel ACA 22 (2.6×100 cm) of ^{125}I -Tg after storage in glycerol 500 g/l at -20°C . The column was eluted with a barbital buffer, 0.075 mol/l, pH 8.5, containing bovine serum albumin, 20 g/l. (a) Tg purified with the addition of protease inhibitors (Tg-PI). Flow rate 12 ml/h. Fraction volume 3.7 ml. (b) Tg purified without the addition of protease inhibitors (Tg-O). Flow rate 15 ml/h. Fraction volume 3.7 ml. 19S denotes the fractions containing the largest proportion of ^{125}I -Tg in the 19S form as determined by polyacrylamide gel electrophoresis.

TABLE 1. Influence of the storage mode and the storage time of ^{125}I -Tg-PI on the intra-assay coefficient of variation as calculated from the standard points and three different controls

Storage mode at -20°C	Time after iodination of Tg (weeks)	Coefficient of variation (%)
50% Glycerol	1	3.9
Buffer	1	8.1
50% Glycerol	6	10.3
Buffer	6	22.9

tration of a sample was doubled and after 5 years it had increased 5 times.

Since the use of glycerol as storage solution for the stock standards was impractical because of the high viscosity of the glycerol solution, and since serum samples had been stored for many years without any apparent change in their Tg content, storage in NGS was tested. This markedly enhanced stability and Tg stored for 3 months showed no tendency to lose potency (Fig. 6, $P>0.5$).

The loss of potency by storage in buffer differed among the various Tg batches, being

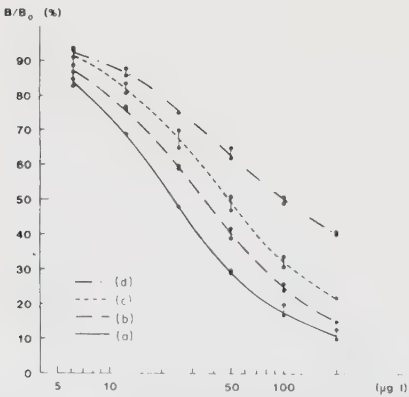


Fig. 5. Dose-response curves for Tg-vH prepared from a stock standard in buffer using antiserum k-7502. (a) Stock standard unfrozen, (b) stock standard frozen at -20°C for 1 month, (c) stock standard frozen at -20°C for 3 months, (d) stock standard frozen at -20°C for 5 years.

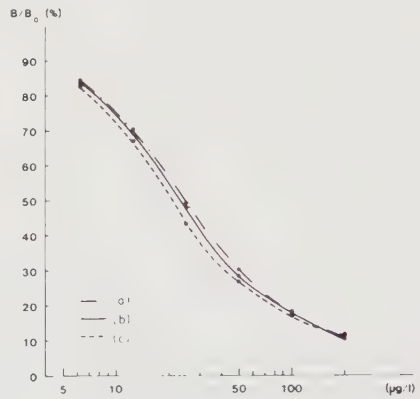


Fig. 6. Dose-response curves for Tg-vH prepared from a stock standard in normal goat serum using antiserum k-7502. (a) Stock standard unfrozen; (b) stock standard frozen at -20°C for 1 month; (c) stock standard frozen at -20°C for 3 months.

most pronounced in Tg purified without the presence of PI (Tg-O and Tg-vH), and least evident in Tg-PI (data not shown).

Antisera

The apparent loss of immunoreactivity of the Tg standard during storage in buffer at -20°C varied with different antisera (Fig. 5 and 7).

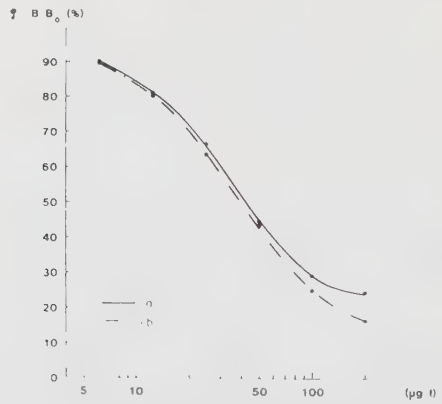


Fig. 7. Dose-response curves for Tg-vH prepared from a stock standard in buffer using antiserum k-8102. (a) Stock standard unfrozen, (b) stock standard frozen in buffer for 3 months.

The progressive loss of immunoreactivity during storage in buffer did not occur when antiserum k-8102 was used instead of antiserum k-7502, not even in the Tg batches prepared without the addition of PI (Tg-O and Tg-vH).

DISCUSSION

Endogenous proteases present in thyroid extracts, which are not entirely removed during the purification, are involved in the degradation of the native 19S Tg [22]. Furthermore, microsomes, membrane particles, etc., which are not removed during the first purification step on the Sephacryl S-200 column, may affect the Tg. During the second purification step on the Sepharose 4B column, however, macroaggregates as well as lower molecular size proteins are removed and therefore do not influence the final results.

Isolation and purification of Tg unprotected against the influence of the thyroid proteases results in Tg with low stability. As the most potent combination of PI previously used (diiso-propyl-fluoro-phosphate, DFP, tosylamide-phenyl-ethyl-chloro-methyl ketone, TPCK, and bipyridyl) did not completely inhibit proteolytic activity [19], we added five more potent protease inhibitors (and used benzamidine instead of bipyridyl) to ensure more effective inhibition. The stability of Tg thus obtained was distinctly enhanced, which improved its usefulness in the Tg-RIA, as compared to Tg isolated without this precaution. The use of Tg-PI also improved the radioiodinated Tg used as tracer in the RIA. It resulted in a constantly higher incorporation of radioiodine than the corresponding Tg purified unprotected by PI (Tg-O and Tg-vH), though the reason for this effect is unclear.

Although the Tg-PI was identified as one distinct peak on gel chromatography immediately after its preparation, it altered rapidly if not stored and handled adequately. The most rapid cleavage occurred after freezing-thawing in buffer, which confirms the findings of Simon *et al.* [28].

The nature of the effect of freezing on proteins in different solvents is unclear. It is assumed that the conformation of the protein alters during freezing, while temperature variations during the frozen stage cause no further change of the conformation [32-33].

The degradation induced by freezing could be avoided by storage of the Tg in 50% glycerol at -20°C . Such a stabilizing effect of glycerol and other polyhydric alcohols has been reported for different proteins [5, 27]. The mechanism of this stabilizing effect is still unclear and may vary from protein to protein.

The conformational changes induced by freezing in buffer also made the molecule more susceptible to degradation by endogenous proteases. On the other hand, preservation of an intact conformation by storage in 50% glycerol minimized degradation, even in the preparation purified without the addition of PI. A practical effect of storage in 50% glycerol was that no repurification of the ^{125}I -labelled Tg was necessary before its use in the assay.

The same stabilizing effect was obtained by freezing the stock standard in serum as by storage in 50% glycerol. That increased protein concentration suppresses the freezing effect has been shown for other large proteins such as phosvitin [32] and immunoglobulin G [9].

The influence of the degradation of Tg on the results of the RIA varied between different antisera. The loss of immunoreactivity, apparent with antiserum k-7502, did not occur when antiserum k-8102 was used. The reason for this variation is difficult to explain, but may be caused by differences in the type of antigenic specificity of the antisera. Antibodies to native globular proteins are directed mostly against conformational rather than sequential determinants [12, 26]. For thyroglobulin the conformational antibodies dominate, that is antibodies directed against sites constructed from discontinuous regions of the protein [16]. Hypothetically, the gradual loss of immunoreactivity during freezing in buffer depends on a decrease of the number of such conformational antigenic determinants.

The results of these studies demonstrate that careful preparation and handling of the Tg is essential to its use in RIA. It is possible that most of the variations in the normal ranges published from different laboratories are caused by lack of caution in handling the rather fragile Tg molecule.

ACKNOWLEDGMENTS

The authors thank Drs R. Ekman and P.

Fernlund for valuable advice and suggestions regarding the manuscript, Ms R. Persson and Mrs A. Murne for excellent technical assistance and Mrs B. Roung for typing the manuscript.

REFERENCES

- 1 Arima, I., Spiro, M.J. & Spiro, R.G. Studies on the carbohydrate units of thyroglobulin. Evaluation of their microheterogeneity in the human and calf proteins. *J. biol. Chem.* **247**, 1825, 1972.
- 2 Bayer, M.F. & Kriss, J.P. Immunoradiometric assay for serum thyroglobulin: semiquantitative measurement of thyroglobulin in antithyroglobulin-positive sera. *J. clin. Endocrinol. Metab.* **49**, 557, 1979.
- 3 Benita, G., Lapalus, F., Bayer, V., Bornet, H. & Hoch, M. Radioimmunoassay of serum thyroglobulin. Technique and clinical results. *Eur. J. nucl. Med.* **6**, 515, 1981.
- 4 Bennett, C.A. & Franklin, N.L. *Statistical Analysis in Chemistry and the Chemical Industry*. John Wiley, Chichester, 1967.
- 5 Bradbury, S.L. & Jakoby, W.B. Glycerol as an enzyme-stabilizing agent: Effects on aldehyde dehydrogenase. *Proc. natn. Acad. Sci. USA* **69**, 2373, 1972.
- 6 Eber, M., Abecassis, J., Grob, J.C., Ott, G. & Methlin, G. Immunoradiometric assay for human thyroglobulin and variations in thyroid pathology. *Clinica chim. Acta* **105**, 51, 1980.
- 7 Endo, Y., Nakano, J., Ohtaki, S., Izumi, M., Hamaguchi, Y., Yoshitake, S. & Ishikawa, E. An enzyme immunoassay for the measurement of thyroglobulin in human serum. *Clinica. chim. Acta* **95**, 325, 1979.
- 8 Feldt-Rasmussen, U. Purification of human thyroglobulin for radioimmunoassay and testing by ultracentrifugal analysis and immunoelectrophoresis. *J. Immunol. Methods* **21**, 295, 1978.
- 9 Hansson, U.-B. Inhibition and reversal of aggregation of immunoglobulin G by freezing. *Acta chem. scand.* **22**, 490, 1968.
- 10 Herle, A.J. van, Uller, R.P., Matthews, N.L. & Brown, J. Radioimmunoassay for measurement of thyroglobulin in human serum. *J. clin. Invest.* **52**, 1320, 1973.
- 11 Ikekubo, K., Jutton, J. & Schneider, A.B. Radioimmunoassay of human thyroglobulin with use of 'thyroglobulin free' plasma prepared by ultracentrifugation is diluent. *Clinica chim. Acta* **26**, 1566, 1980.
- 12 Lerner, R.A. Mapping the immunological repertoire to produce antibodies of predetermined specificity. *Nature* **299**, 592, 1982.
- 13 Lissitzky, S., Roques, M., Forresam, J. & Simon, C. Biosynthese. Iodation et Hétérogénéité de la Thyroglobuline. *Bull. Soc. Chim. Biol.* **47**, 1999, 1965.
- 14 Lowry, O.H., Rosebrough, N.J., Farr, A.I. & Randall, R.T. Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265, 1951.
- 15 Mariotti, S., Cupini, C., Giani, C., Lari, R., Rolletti, E., Falco, A., Marchisio, M. & Pinchera, A. Evaluation of a solid-phase immunoradiometric assay (IRMA) for serum thyroglobulin: effect of anti-thyroglobulin autoantibody. *Clinica chim. Acta* **123**, 347, 1982.
- 16 Metzger, H., Sharp, G.C. & Edelhoch, H. The properties of thyroglobulin VII. The immunologic activity of thyroglobulin fragments. *Biochemistry* **1**, 205, 1962.
- 17 Ochi, Y., Hachiya, I., Yoshimura, M., Miyazaki, T., Majima, T., Kamatsu, I. & Takahashi, H. Radioimmunoassay for estimation of thyroglobulin in human serum. *Endocrinol. Jpn* **22**, 351, 1975.
- 18 Pezzino, V., Cozzani, P., Filetti, S., Galbiati, A., Lisi, E., Squatrito, S. & Vigneri, R. A radioimmunoassay for human thyroglobulin: methodology and clinical applications. *Eur. J. clin. Invest.* **7**, 503, 1977.
- 19 Pitt-Rivers, R. *Molecular Mechanisms and Regulation of Thyroid Hormone Biosynthesis* vol. 1, pp. 47-49, Accademia Nazionale dei Lincei, Roma, 1975.
- 20 Ratcliffe, J.G., Ayoub, L.A.W. & Pearson, D. The measurement of serum thyroglobulin in the presence of thyroglobulin antibodies. *Clin. Endocrinol.* **15**, 507, 1981.
- 21 Rolland, M. & Lissitzky, S. Polypeptides noncovalently associated in 19S thyroglobulin. *Biochim. biophys. Acta* **278**, 316, 1972.
- 22 Rolland, M. & Lissitzky, S. Endogenous proteolytic activity and constituent polypeptide chains of sheep and pig 19S thyroglobulin. *Biochim. biophys. Acta* **427**, 696, 1976.
- 23 Roti, E., Robuschi, G., Emanuele, R., Bandini, P. & Gnudi, A. Radioimmunoassay of thyroglobulin in human serum: concentrations in normal subjects and in patients with thyroid disease. *J. Nucl. Med. Allied Sci.* **25**, 57, 1981.
- 24 Schneider, A.B. & Ikekubo K. Measurement of thyroglobulin in the circulation: clinical and technical considerations. *Ann. clin. Lab. Sci.* **9**, 230, 1979.
- 25 Schneider, A.B. & Pervos, R. Radioimmunoassay of human thyroglobulin: Effect of antithyroglobulin autoantibodies. *J. clin. Endocrinol. Metab.* **47**, 126, 1978.
- 26 Sela, M., Schechter, B., Schechter, I. & Borek, F. Antibodies to sequential and conformational determinants. *Cold Spring Harbor Symp. Quant. Biol.* **32**, 537, 1967.
- 27 Shafir, S. & Parrott, C.L. Influence of glycerol and other polyhydric alcohols on the quaternary structure of an oligomeric protein. *Archs Biochem. Biophys.* **166**, 426, 1975.
- 28 Simon, C., Roques, M., Forresam, J. & Lissitzky, S. Effect of propylthiouracil on the iodination and maturation of rat thyroglobulin. *Acta endocrinol.* **53**, 271, 1966.
- 29 Spiro, M.J. Evaluation of the homogeneity of several thyroglobulin preparations. *J. biol. Chem.* **236**, 2901, 1961.

- 30 Spiro, M.J. Subunit heterogeneity of thyroglobulin *J. biol. Chem.* **248**, 4446, 1973.
- 31 Svendsen, P.J. & Axelsen, N.H. A modified antigen-antibody crossed electrophoresis characterizing the specificity and titre of human precipitins against *Candida albicans* *J. Immunol. Methods* **1**, 169, 1972.
- 32 Taborsky, G. Effect of freezing and thawing on the conformation of phosvitin *J. biol. Chem.* **245**, 1054, 1970.
- 33 Taborsky, G. Solute redistribution in some multi-component aqueous systems on freezing *J. biol. Chem.* **245**, 1063, 1970.
- 34 Thorell, J.I. & Johansson, B.G. Enzymatic iodination of polypeptides with ^{125}I to high specific activity *Biochim. biophys. Acta* **251**, 363, 1971.
- 35 Torrigiani, G., Doniach, D. & Rott, I.M. Serum thyroglobulin levels in healthy subjects and in patients with thyroid disease *J. clin. Endocrinol. Metab.* **29**, 305, 1969.
- 36 Vaitukaitis, J., Robbins, J.B., Nieschlag, E. & Ross, G.T. A method for producing specific antisera with small doses of immunogen *J. clin. Endocrinol. Metab.* **33**, 988, 1971.

Received 4 November 1983

Accepted 10 February 1984

A new sensitive immunosorbent radioassay for the detection of circulating antibodies to polypeptide hormones and proteins

U.-B. ERICSSON, I. LARSSON, A. MURNE & J. I. THORELL

Departments of Internal Medicine and Clinical Chemistry, Malmö General Hospital, University of Lund, Malmö, Sweden

Ericsson, U.-B., Larsson, I., Murne, A. & Thorell, J.I. A new sensitive immunosorbent radioassay for the detection of circulating antibodies to polypeptide hormones and proteins. *Scand. J. clin. Lab. Invest.* 44, 000-000, 1984.

A solid-phase immunosorbent radioassay for the detection of circulating antibodies to protein hormones is described. The assay is based on the binding of the homologous 125 I-labelled antigen to the antibodies which are then bound to anti-IgG antibodies covalently coupled to Sepharose. It can easily be applied as a complement to any radioimmunoassay for the detection of circulating antibodies to the ligand measured. The assay system avoids falsely elevated values due to interference of high serum concentrations of the antigen. The assay was applied to measure antibodies to FSH, LH, TSH, GH, prolactin, insulin and thyroglobulin (Tg). Among patients with chronic thyroiditis Tg antibodies were found in 100% of the sera. In diffuse toxic goitre 73% of the patients had detectable Tg antibodies. Insulin antibodies were present in 82% of the sera from patients with insulin treated diabetes. No antibodies were found against the other protein hormones tested.

Key-words: radioimmunoassay; antibodies; thyroglobulin; insulin; protein hormones

Ulla-Britt Ericsson, Department of Internal Medicine, Malmö General Hospital, S-214 01 Malmö, Sweden

The occurrence of circulating antibodies to hormonal polypeptides has been reported both for exogenous and endogenous antigens. It was first revealed for such highly immunogenic substances as thyroglobulin (Tg) by Roitt *et al.*, 1956 [25], who demonstrated, in the serum of patients with Hashimoto's disease, precipitating antibodies which reacted specifically with saline extract of thyroid tissue. Berson & Yalow [4], in their pioneering work on radioimmunoassay methodology, revealed that proteins of smaller molecular size, like insulin, could also induce antibodies in a high frequency. They also showed that antibodies to insulin may interfere with the

assay and prevent the accurate determination of insulin in these patients. A similar source of error has been recognized in the radioimmunoassay (RIA) for Tg [27].

Low concentrations of autoantibodies to Tg appear in a significant number of asymptomatic individuals [2, 7, 13, 14, 17, 32]. The recognition of these antibodies is a useful marker of autoimmune disease [1, 5, 18, 21, 29, 34]. Furthermore, the clinical assessment of resistance to insulin and to GH treatment is facilitated by the determination of insulin and GH antibodies [10, 16, 20, 31].

We therefore developed a sensitive

immunosorbent radioassay which detects the binding of ^{125}I -labelled antigen to antibodies isolated with an immunosorbent of IgG antibodies coupled to Sepharose. This simple assay can be applied to detect antibodies to any hormone for which a labelled antigen is available at the laboratory. We describe here its application for the screening of antibodies to insulin, FSH, LH, TSH, GH, prolactin (Prl) and Tg in healthy individuals and in patients with various diseases.

MATERIALS AND METHODS

Samples

Sera from patients and controls were kept frozen at -20°C until analysed.

Controls

Twenty-nine apparently healthy individuals (23 women, 6 men, aged 22–63 years, median 37 years) from the laboratory staff served as controls.

Patients

Sera were obtained from the following patient groups:

Insulin antibodies. Thirty-nine patients with insulin-treated diabetes (20 women, 19 men, aged 8–71 years, median 18 years) were tested.

FSH and LH antibodies. Twenty women (aged 30–72 years, median 54 years) with high serum levels of FSH and/or LH were analysed.

GH antibodies. Eighteen patients with acromegaly (10 women, 8 men, aged 25–70 years, median 53 years) with varying degrees of elevated GH concentrations were analysed.

TSH antibodies. Twenty-five patients (16 women, 9 men, aged 22–90 years, median 60 years) with elevated TSH concentrations were tested.

Prl antibodies. Sera from 24 women (aged 17–71 years, median 34 years) with varying degrees of hyperprolactinaemia, were analysed.

Tg antibodies. Fifteen patients with toxic diffuse goitre (12 women, 3 men, aged 18–73 years, median 46 years) were tested. For comparison 28 patients with chronic thyroiditis (27 women, 1 man, aged 29–88 years, median 60.5 years) were studied.

Antigens

Human thyroglobulin was purified as described in detail elsewhere [8]. Porcine insulin was from Novo (Copenhagen, Denmark). FSH, LH, TSH, GH and Prl were from KABI (Stockholm, Sweden).

Antisera

Goat anti-human IgG used in the assay was prepared by immunizing goats with human IgG (KABI, Stockholm, Sweden). Ten milligrams of IgG in 2 ml 0.9% NaCl were emulsified with 2 ml of Freund's complete adjuvant (Difco laboratories, Detroit, Mich., USA). Each goat received 10 mg of IgG injected by multiple site subcutaneous injections. Booster doses were given after 6 and 12 months. Antiserum was harvested 3 weeks after the last injection. The goat anti-human IgG was then separated by salting out with 33% saturated ammonium sulphate and dialysed against NaHCO_3 , 0.1 mol/l, for 48 h at $+4^{\circ}\text{C}$.

Buffer

The buffer used in the assay for dilution of the samples and the labelled antigen was a barbital buffer, 0.075 mol/l, pH 8.6, containing 0.025% bovine serum albumin, 0.025% EDTA and 0.05% NaN_3 (buffer A). The same buffer with 1% Tween 20 (buffer B) was used for suspending the Sepharose-anti-IgG and for washing.

Antigen sorbent

Goat anti-human IgG from 100 ml serum was linked to 30g CNBr activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) following the description of the manufacturer. The sorbent was diluted in 4×50 ml barbital buffer, pH 8.6, containing 0.05% NaN_3 and then dispersed into particles of 1–20 μm by treatment for 8 min at 20 000 rpm with a polytron (PT 10 ST 'OD' S, 12 mm, Kinematica, Luzerne, Switzerland). The sorbent was then centrifuged and decanted and resuspended in buffer B to a final volume of 500 ml.

Radiolabelled antigens

The protein hormones were iodinated with Na^{125}I (Code IMS 30, Amersham International,

Amersham, England) with the chloramine-T method [19]. Insulin and LH were iodinated with the lactoperoxidase method [30]. All radiolabelled proteins were diluted to a specific activity of 1000 Bq/ml (20 000 cpm).

Insulin and LH. Five micrograms of the antigen were iodinated with 37 MBq ^{125}I (specific activity: 5.9–6.7 MBq/ μg). The iodinated protein was then separated from unbound ^{125}I by adsorption chromatography on cellulose (Whatman CF 11).

FSH, GH and TSH. Five micrograms of the antigen were iodinated with 37 MBq ^{125}I (specific activities: FSH, 4.4–5.9 MBq/ μg ; GH, 5.2–6.4 MBq/ μg ; TSH, 5.9–6.7 MBq/ μg). The iodinated protein was purified with gel chromatography on a 12 $\times$ 1 cm column of Sephadex G-50 (Pharmacia Fine Chemicals, Uppsala, Sweden).

Prl. Five micrograms Prl were iodinated with 9.75 MBq ^{125}I (specific activity: 0.8–1.1 MBq/ μg). The iodinated protein was purified by gel filtration on a 16 $\times$ 1 cm column of Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden).

Tg. Ten micrograms Tg were iodinated with 18.5 MBq ^{125}I (specific activity: 1.30–1.48 MBq/ μg). The iodinated protein was then separated from unbound ^{125}I by gel filtration on a 15 $\times$ 1 cm column of Sephadex G-200 (Pharmacia Fine Chemicals, Uppsala, Sweden).

Assay

The tests were performed in 11 $\times$ 55 mm polystyrene tubes. Each sample was assayed undiluted and diluted 1:5, 1:25 and 1:125. One hundred μl of the sample were mixed (Vortex) with 400 μl of the ^{125}I -labelled antigen (final dilutions 1:5, 1:25, 1:125 and 1:625) and incubated overnight at +4°C. Fifty μl of the incubate were mixed with 250 μl of the Sepharose anti-human IgG suspension and incubated 2 h at room temperature. To each tube one ml of the diluent was added and the tubes were centrifuged 15 min at 2400 g and +4°C and decanted. The radioactivity was measured for 5 min in a multi-crystal gamma counter (Nuclear Enterprises, NE 1600, Edinburgh, Scotland). Each assay series included a negative control serum.

In the Tg antibody assay 25 μl of normal goat serum were added during the final incubation period.

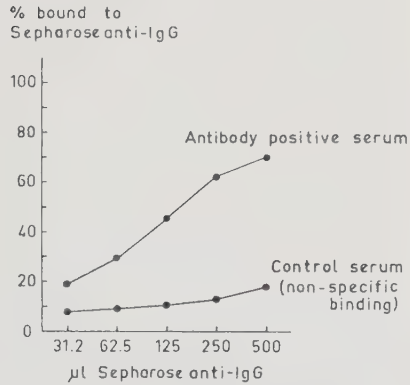


Fig. 1. Effect of the amount of Sepharose-anti-IgG on the specific and nonspecific binding in the insulin antibody assay. The antibody positive serum was from an insulin-treated diabetic patient and the control serum from a healthy person who had never received insulin.

All results were expressed as a percentage of ^{125}I -antigen bound. All samples were assayed in serial dilution until the binding reached the detection limit (the 97.5th percentile). One dilution step (final dilution) before this stage was taken as the titre.

RESULTS

Assay characteristics

Of all the radiolabelled antigens tested, an amount of 250 μl of the Sepharose anti-IgG reagent gave the highest binding in sera containing antibodies (sera from insulin treated diabetic patients) and the lowest nonspecific binding in sera without antibodies (healthy controls who have never received insulin). Thus, this amount gave the best separation between sera with and without antibodies (Fig. 1).

In sera containing antibodies, the specific binding showed a typical pattern with progressively decreased binding at increased dilution of the serum. In sera not containing specific antibodies the binding of ^{125}I -antigen either did not change or increased at higher dilutions of the serum.

The nonspecific binding was the same for all the radiolabelled antigens tested (approximately

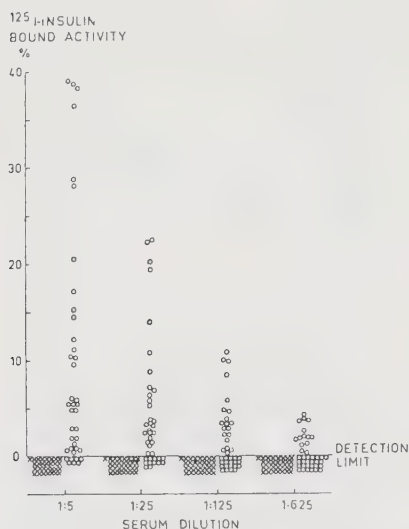


FIG. 2. Binding of ^{125}I -insulin in the insulin antibody assay at different dilutions of the same serum sample (final dilution) in healthy controls (x) and in patients with insulin treated diabetes (o). The ^{125}I -insulin bound activities are corrected for the nonspecific binding.

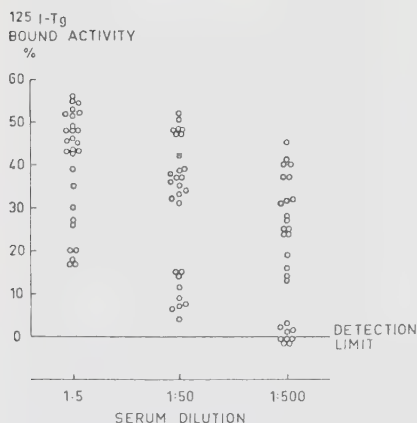


FIG. 3. Binding of ^{125}I -thyroglobulin in the thyroglobulin antibody assay at three different dilutions of the same serum sample (final dilution) in patients with chronic thyroditis. The ^{125}I -thyroglobulin bound activities are corrected for the nonspecific binding.

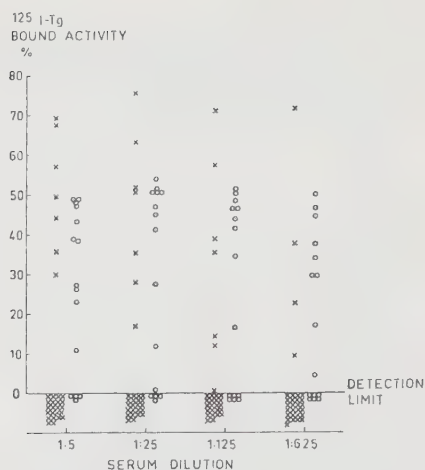


FIG. 4. Binding of ^{125}I -thyroglobulin in the thyroglobulin antibody assay at different dilutions of the same serum sample (final dilution) in healthy controls (x) and in patients with thyrotoxicosis (o). The ^{125}I -thyroglobulin bound activities are corrected for the nonspecific binding.

15%). Its upper limit (the 97.5th percentile) was established from sera which showed a low and unchanged or gradually increased binding at successive dilutions of the sera. The level of the nonspecific binding was controlled in each assay run.

The statistical methods available to calculate the intra- and inter-assay coefficients of variation are not applicable in an assay system like this, where the results are given in titres. Therefore, coefficients of variation are not given. In 4/31 duplicate determinations of antibody positive samples in the Tg antibody assay and in 8/71 duplicate determinations of antibody positive samples in the insulin antibody assay, the titres differed by one dilution step. The titre of the insulin antibody positive sample (1:625) used as control serum in the insulin antibody assay was the same in 13 assays run over 1 year.

Antibody determination

Insulin. (Fig. 2). Thirty-two out of 39 patients (82%) with insulin treated diabetes were antibody positive, whereas none of the controls had a

detectable antibody binding. Of the antibody positive sera 44% had a titre of 1:625 or more.

FSH, LH, TSH, GH and Prl. All sera tested, from patients with high serum concentrations of the respective hormones as well as the controls, showed a binding below the detection limit. Thus, all sera were regarded as antibody negative.

Tg. All 28 patients with chronic thyroiditis had a binding much above the detection limit. Their titres were higher than those seen in any other patient group (Fig. 3). Eleven out of 15 (73%) of the patients with toxic diffuse goitre and 7 out of 29 (24%) of the controls had a binding above the detection limit (Fig. 4).

DISCUSSION

Organ-specific autoantibodies are frequently found in patients with various endocrinopathies [33] and systemic diseases [28], but they are also found in clinically healthy individuals [2, 7, 13, 14, 17, 32]. Immunofluorescent and haemagglutination methods are commonly used for the detection of such antibodies [6, 12]. Several alternative techniques have now become available e.g. various radioligand assays. The radio-immunological methods are both specific and sensitive. They are more specific than the immunofluorescent techniques and more sensitive than the haemagglutination methods. Most RIA methods also permit the results to be expressed in quantitative terms. However, radioassays based on a competitive binding are likely to produce false positive or false negative results in the presence of abnormal concentrations of the antigen [22, 24]. For example, the presence of an excess of the endogenous homologous antigen in the serum competes with the labelled antigen and prevents the detection of antibodies present in low concentration [3].

Another disadvantage is the varying non-specific binding sometimes found in double-antibody radioimmunoassays. Peak *et al.* [23] found, in an assay for Tg antibodies, that the nonspecific coprecipitation of radioactive Tg with normal serum could be decreased by 50–70% by adsorbing the ^{125}I -Tg with normal serum and second antibody prior to its use in the assay. The mechanism behind this effect and the nature of the labelled material removed during adsorption is still unclear. We tested a solid-phase immunosorbent radioassay which involved

antigen coupled to Sepharose [15]. The binding of antibodies to this antigen was detected with ^{125}I -Protein-A. When it was adopted for the assay of Tg antibodies, we found a high and variable binding, which could be removed by pretreatment of the samples with uncoupled Sepharose. The degree of nonspecific binding was directly dependent on the amount of Sepharose used for pretreatment. Because of the high nonspecific binding in such assay systems, undiluted serum samples could not be assayed, thereby reducing the sensitivity.

With the solid-phase radioassay system described here, falsely elevated values, because of interference with high serum concentrations of the antigen, are precluded. With this assay we found insulin antibodies in 82% of the insulin treated diabetics, which is in agreement with the results of Kurtz *et al.* [20] and Heding *et al.* [16] for patients treated with highly purified porcine insulin.

The assay system described here can be used to measure auto-antibodies to many other proteins and peptides with an equally high level of sensitivity. When applied to detect Tg antibodies, we found, in agreement with Feldt-Rasmussen *et al.* [9] that all patients with chronic thyroiditis had detectable Tg antibodies. Peak *et al.* [23] and Mori & Kriss [22] found antibodies in 95% of their patients with chronic thyroiditis. Like others who use sensitive RIA methods, we found Tg antibodies in over 70% of the patients with toxic diffuse goitre [22, 23]. Tg antibodies were also found in a high frequency of apparently healthy controls, whereas no insulin antibodies were found in the same control group.

Antibodies to TSH and GH have been described previously in individuals who have received TSH and GH preparations [10, 11, 26, 31]. To our knowledge, spontaneous endogenous auto-antibodies to the pituitary hormones have not been described. Also, with the sensitive assay used in this study all patient and control sera were negative with respect to FSH, LH, TSH, GH and Prl antibodies. On the other hand, the whole spectrum from very high to no detectable antibodies were found in the insulin and Tg antibody assays, in the presence of low- as well as high-serum concentrations of the respective antigen. Therefore, the possibility that the negative results, with respect to antibodies to the other antigens tested, were caused by the saturation of

the antibodies by high serum concentrations of the endogenous hormone, is most unlikely.

The reason for utilizing two different methods of radioiodination of the antigen was an unexplained variation in the assay of antibodies to thyroglobulin and prolactin which was obviated when the lactoperoxidase labelling procedure was substituted by the chloramine-T method. Further studies have revealed that this was caused by the occurrence of antibodies to lactoperoxidase in a high frequency in the normal population. These antibodies interfered with the assay when the method used for purification of the radioiodinated antigen did not remove the traces of the lactoperoxidase remaining after the iodination procedure. These results are to be reported in detail elsewhere.

REFERENCES

- Anderson, J.W., McConahey, W.M., Alarcón-Segovia, D., Emslander, R.F. & Wakim, K.G. Diagnostic value of thyroid antibodies. *J. clin. Endocrinol. Metab.* 27, 937, 1967.
- Assem, E.S.K., Trotter, W.R. & Belya-vin, G. Thyroglobulin and thyroglobulin antibodies in the serum of normal adults. *Immunology* 9, 21, 1965.
- Bayer, M.F. & Kriss, J.P. A solid phase sandwich-type radioimmunoassay for antithyroglobulin: elimination of false positive results and semi-quantitative measurement of antithyroglobulin in the presence of elevated thyroglobulin. *J. clin. Endocrinol. Metab.* 49, 565, 1979.
- Berson, S.A., Yalow, R.S., Bauman, A., Rothschild, M.A. & Newerly, K. Insulin-¹²⁵I metabolism in human subjects: demonstration of insulin binding globulin in the circulation of insulin treated subjects. *J. clin. Invest.* 35, 170, 1956.
- Burek, C.L., Hoffman, W.H., & Rose, N.R. The presence of thyroid autoantibodies in children and adolescents with autoimmune thyroid disease and in their siblings and parents. *Clin. Immunol. Immunopathol.* 25, 395, 1982.
- Coons, A.H. & Kaplan, M.H. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. *J. exp. Med.* 91, 1, 1950.
- Dingle, P.R., Ferguson, A., Horn, D.B., Tubmen, J. & Rall, R. The incidence of thyroglobulin antibodies and thyroid enlargement in a general practice in north-east England. *Clin. exp. Immunol.* 1, 277, 1966.
- Ericsson, U.-B., Larsson, I. & Thorell, J.I. Purification and storage of thyroglobulin. Two important factors influencing the radioimmunoassay for thyroglobulin. *Scand. J. clin. Lab. Invest.* 44, 1984 (in press).
- Feldt-Rasmussen, U., Perrild, H., Bech, K., Bliddal, H., Date, J., Høier Madsen, M., Nordfang, O., Ryder, L.P., Thomsen, M., Kappelgaard, E. & Nielsen, H. Discrepancy between haemagglutination and radioimmunological techniques for measurement of serum thyroglobulin auto-antibodies. *Allergy* 38, 49, 1983.
- Frasier, D.S. & Smith, F.G., Jr. Antibodies to human growth hormone. *Am. J. Dis. Child.* 112, 383, 1966.
- Frohman, L.A., Baron, M.A. & Schneider, A.B. Plasma immunoreactive TSH: spurious elevation due to antibodies to bovine TSH which cross-react with human TSH. *Metabolism* 31, 834, 1982.
- Fulthorpe, A.J., Roitt, I.M., Doniach, D. & Couchman, K. A stable sheep cell preparation for detecting thyroglobulin auto-antibodies and its clinical applications. *J. clin. Pathol.* 14, 654, 1961.
- Gordin, A., Maatela, J., Miettinen, A., Helenius, T. & Lamberg, B.-A. Serum thyrotrophin and circulating thyroglobulin and thyroid microsomal antibodies in a Finnish population. *Acta endocrinol.* 90, 33, 1979.
- Hackett, E., Beech, M. & Forbes, I.J. Thyroglobulin antibodies in patients without clinical disease of the thyroid gland. *Lancet* ii, 402, 1960.
- Hamilton, R.G., Rendell, M. & Adkinson, N.F. Serological analysis of human IgG and IgE anti-insulin antibodies by solid-phase radioimmunoassays. *J. Lab. clin. Med.* 96, 1022, 1980.
- Heding, L.G., Larsson, Y. & Ludvigsson, J. The immunogenicity of insulin preparation. Antibody levels before and after transfer to highly purified porcine insulin. *Diabetologia* 19, 511, 1980.
- Hill, O.W. Thyroglobulin antibodies in 1297 patients without thyroid disease. *Br. Med. J.* 1, 1793, 1961.
- Hopwood, N.J., Rabin, B.S., Foley, T.P., Jr. & Peak R.L. Thyroid antibodies in children and adolescents with thyroid disorders. *J. Pediatr.* 93, 57, 1978.
- Hunter, W.M. & Greenwood, F.C. Preparation of ¹²⁵I-iodine-131 labelled human growth hormone of high specific activity. *Nature* 194, 495, 1962.
- Kurtz, A.B., Matthews, J.A., Mustaffa, B.E., Daggett, P.R. & Nabarro, J.D.N. Decrease of antibodies to insulin, proinsulin and contaminating hormones after changing treatment from conventional beef to purified pork insulin. *Diabetologia* 18, 147, 1980.
- Maagøe, H., Reintoft, I., Christensen, H.E., Simonsen, J. & Mogensen, E.F. Lymphocytic thyroiditis. I. Correlation between morphological, immunological and clinical findings. *Acta med. scand.* 201, 299, 1977.
- Mori, T. & Kriss, J.P. Measurement by competitive binding radioassay of serum anti-microsomal and anti-thyroglobulin antibodies in Graves' disease and other thyroid disorders. *J. clin. Endocrinol. Metab.* 33, 688, 1971.
- Peake, R.L., Willis, D.B., Asimakis, G.K., Jr., & Deiss, W.P., Jr. Radioimmunologic assay for anti-thyroglobulin antibodies. *J. Lab. clin. Med.* 84, 907, 1974.
- Pinchera, A., Mariotti, S., Vitti, P., Tosi, M., Grasso, L., Pacini, F., Buti, R. & Baschieri, L.

- Interference of serum thyroglobulin in the radioassay for serum antithyroglobulin antibodies. *J. clin. Endocrinol. Metab.* **45**, 1077, 1977.
- 25 Roitt, I.M., Doniach, D., Campbell, P.N. & Hudson, R.V. Auto-antibodies in Hashimoto's disease (lymphadenoid goitre). *Lancet* **ii**, 820, 1956.
 - 26 Sain, A., Sham, R., Singh, A. & Silver, L. Erroneous thyroid-stimulating hormone radioimmunoassay results due to interfering antiovine thyroid-stimulating hormone antibodies. *Am. J. clin. Pathol.* **71**, 540, 1979.
 - 27 Schneider, A.B. & Pervos, R. Radioimmunoassay of human thyroglobulin: effect of antithyroglobulin autoantibodies. *J. clin. Endocrinol. Metab.* **47**, 126, 1978.
 - 28 Skanse, B. & Nilsson, S.-B. Hypergammaglobulinemia and thyroid antibodies. *Acta med. scand.* **172**, 505, 1962.
 - 29 Tanner, A.R., Scott-Morgan L., Mardell R. & Lloyd R.S. The incidence of occult thyroid disease associated with thyroid antibodies identified on routine autoantibody screening. *Acta endocrinol.* **100**, 31, 1982.
 - 30 Thorell, J.I. & Johansson, B.G. Enzymatic iodination of polypeptides with ^{125}I to high specific activity. *Biochim. biophys. Acta* **251**, 363, 1971.
 - 31 Waldhäusl, W.K. & Rath, F. Development of antibodies to human growth hormone (HGH) in children of short stature treated with HGH. *Acta endocrinol.* **68**, 345, 1971.
 - 32 Whittingham, S., Irwin, J., Mackey I.R., Marsh, S. & Cowling, D.C. Autoantibodies in healthy subjects. *Aust. NZ Ann. Med.* **18**, 130, 1969.
 - 33 Volpé, R. The role of autoimmunity in hypoendocrine and hyperendocrine function. With special emphasis on autoimmune thyroid disease. *Ann. Intern. Med.* **87**, 86, 1977.
 - 34 Yoshida, H., Amino, N., Yagawa, K., Uemura, K., Satoh, M., Miyai, K. & Kumahara, Y. Association of serum antithyroid antibodies with lymphocytic infiltration of the thyroid gland: studies of seventy autopsied cases. *J. clin. Endocrinol. Metab.* **46**, 859, 1978.

Received 22 December 1983

Accepted 6 March 1984

Interference of Endogenous Lactoperoxidase Antibodies in a Solid-Phase Immunosorbent Radioassay for Antibodies to Protein Hormones

by

U-B Ericsson and I Larsson

Departments of Internal Medicine and Clinical Chemistry, Malmö
General Hospital, University of Lund, Malmö Sweden

ABSTRACT

Interference of endogenous lactoperoxidase (LPO) antibodies has been demonstrated in a solid-phase immunosorbent radioassay for the detection of protein hormone antibodies. On enzymatic iodination with LPO, a few per cent of the ^{125}I is incorporated in the enzyme itself by self-iodination. Since the ^{125}I -LPO is not removed from the tracer by chromatography on Sephadex G-50, G-100 and G-200 columns, endogenous antibodies to LPO, which were present in 25/28 sera from healthy individuals, were detected in conjunction with the measurement of antibodies to GH, prolactin and thyroglobulin, causing "false" positive results. Contaminating radioactive LPO could be removed by purification with adsorption chromatography on cellulose and with high performance liquid chromatography, and can be avoided by the use of iodination with immobilized LPO or with chloramine-T.

INTRODUCTION

Enzymatic iodination with lactoperoxidase (LPO) is a method widely used for the radioactive labelling of proteins. LPO has been found to be mild in its effects on the proteins which thus retain their immunological reactivity after iodination (1). During the iodination procedure, a few per cent of the iodine is incorporated in the LPO by self-iodination (2). Since this auto-labelled LPO is not always eliminated during the purification step following the iodination, it may affect radioimmunoassay results.

During the development of a solid-phase immunosorbent radioassay for antibody detection, a high bound activity was found in many samples not expected to contain antibodies, when the tracer was labelled with

the LPO method but not when it was labelled with the chloramine-T method. This "false" binding was found to be caused by endogenous antibodies to bovine LPO.

MATERIALS AND METHODS

Samples

Serum samples from apparently healthy members of the laboratory staff were analyzed with respect to antibodies to growth hormone (GH), prolactin (PrI), insulin, thyroglobulin (Tg) or lactoperoxidase (LPO).

Antigens

Human Tg was purified as described in detail elsewhere (3). GH and PrI were obtained from KABI (Stockholm, Sweden), and porcine insulin from Novo (Copenhagen, Denmark). LPO was purified from unpasteurized cow milk (1). The purified LPO had an $A_{412\text{nm}}/A_{280\text{ nm}}$ ratio of 0.8-0.9.

Radiolabelled antigens

GH, PrI, insulin and Tg were iodinated with $\text{Na-}^{125}\text{I}$ (code IMS 30, Amersham International, Amersham, England), using the lactoperoxidase method (1). GH, PrI and Tg were also iodinated with the chloramine-T method (4). The iodination and purification procedures were performed as described in detail elsewhere (3). The amounts of antigen and $\text{Na-}^{125}\text{I}$ used were the same for both labelling procedures. The following purification methods were used: Gel chromatography on Sephadex G-50 (GH), Sephadex G-100 (PrI) and Sephadex G-200 (Tg), adsorption chromatography on a 2 x 1 cm column of cellulose (Whatman CF 11) (5) or high performance liquid chromatography (HPLC) (GH and PrI) on an Ultro Pac TSK-G 2000 SW column (7.5 x 600 mm; LKB, Sweden). The pump for the liquid chromatography was an LKB 2150 pump with a ceramic head; the column was eluted with phosphate-buffered saline ($\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 0.1 mol/L; NaCl , 0.1 mol/L, pH 6.80) at a flow rate of 0.25 mL/min.

LPO

The LPO was labelled with Na- ^{125}I by self-iodination. The following reagents were added to an 11 x 55 mm polystyrene tube under continuous stirring: 1.0 mCi (37 MBq) Na- ^{125}I (10-12 μL), 10 μg LPO (25 μL , 0.4 mg/mL in phosphate buffer, 0.05 mol/L, pH 7.5), 0.09 μg H_2O_2 (3 μL , 0.03 g/L in distilled water). After 60 s, 0.5 mL phosphate buffer, 0.05 mol/L, pH 7.5 was added. Unreacted iodide was removed by gel chromatography on a 15 x 1 cm column of Sephadex G-50 that had been swollen in barbital buffer (0.075 mol/L, pH 8.6). The column was pretreated with 1.0 mL bovine serum albumin (20 g/L) to prevent adsorption of the labelled enzyme. Excess albumin was eluted with 30 mL of the barbital buffer above. One mL fractions were collected into 1.0 mL of barbital buffer containing bovine serum albumin (20g/L). The yield was calculated from the relative distribution of radioactivity in the protein peak (void volume) and the salt peak. The yield was 50-56% and the specific activity was 0.92-1.03 MBq/ μg . The ^{125}I -labelled LPO was stored at -20°C until used.

Assay

The assay was performed as described in detail elsewhere (6). One hundred microlitre serum was mixed with 400 μL of the ^{125}I -labelled antigen and incubated overnight at $+4^\circ\text{C}$. Fifty microlitres of the incubate was mixed with 250 μL of a Sepharose-anti-human IgG suspension and incubated 2 h at room temperature. To each tube one mL barbital buffer, 0.075 mol/L, pH 8.6 with bovine serum albumin, 20 g/L, was added and the tubes were centrifuged 15 min at $2400 \times g$ and $+4^\circ\text{C}$, decanted and the radioactivity of the precipitate was measured.

The results were expressed as the bound ^{125}I -antigen in per cent. All samples were assayed in serial dilutions.

Statistical methods

Wilcoxon's test was used for paired observations. The co-variation was calculated with the use of the product-moment correlation coefficient.

RESULTS

Fig. 1 illustrates the results of the measurements of endogenous antibodies to bovine LPO. Such antibodies were found in 25/28 serum samples. In 12/28 samples the binding was above 50%.

The specificity of this binding was tested by the addition of unlabelled bovine LPO and unlabelled IgG in increasing concentrations to an antibody positive and an antibody negative serum. Inhibition of the binding of ^{125}I -LPO was observed in the antibody positive serum with both unlabelled LPO and unlabelled IgG, while in the antibody negative serum the binding of ^{125}I -LPO changed very little (Table I). The influence of these antibodies in three different antibody assays was shown by comparing the antibody binding of the antigen iodinated with the LPO method with that of the chloramine-T method (Fig. 2); for all three antigens tested, the binding of the ^{125}I -antigen to the antibodies was significantly higher when the antigen was iodinated with the LPO method ($p < 0.01$). In the insulin antibody assay, in which the ^{125}I -insulin is purified by adsorption chromatography on cellulose, a separation method which removes the contaminating ^{125}I -LPO, this effect was not observed (data not shown).

The influence of the endogenous LPO antibodies in the antibody assay was studied by comparing the supposed falsely high values for the specific ^{125}I -antigen bound, caused by the presence of ^{125}I -LPO in

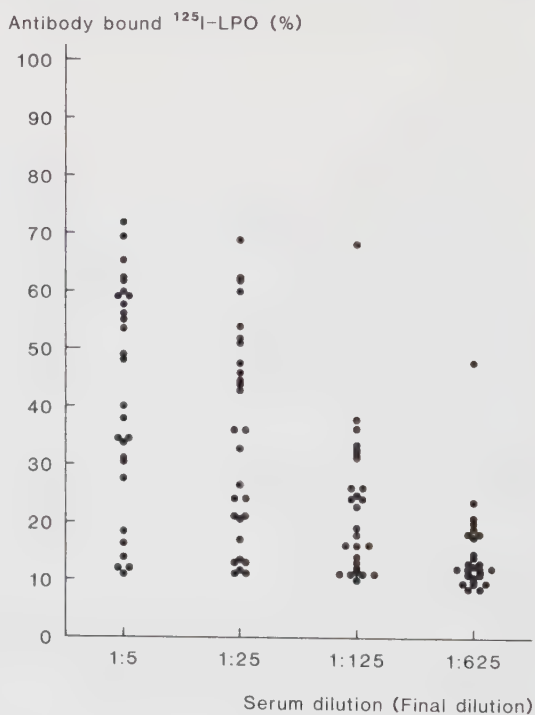


Fig. 1. Antibody binding of ^{125}I -lactoperoxidase (LPO) in different dilutions of the same serum samples (final dilutions) from 28 healthy individuals.

TABLE I. Inhibition of the binding of ^{125}I -LPO in the LPO antibody radioassay with the addition of unlabelled LPO or unlabelled IgG.

Serum sample	Amount of unlabelled LPO added (g/L)		Amount of unlabelled IgG added (g/L)	
	0	0.01	0	20
Antibody positive, % bound activity	44.6	11.1	53.4	26.9
Antibody negative, % bound activity	10.9	10.0	12.7	12.6

each sample (Fig. 3). The highly significant correlation found for all three antigens studied (GH: $r = 0.88$, Prl: $r = 0.90$ and Tg: $r = 0.95$; $p < 0.001$ for all of them), verifies the influence of the LPO antibodies in the assays.

On chromatography of the ^{125}I -Tg, iodinated with LPO, on a Sephadex G-200 column (15 x 1 cm), the iodinated Tg moves with the void volume

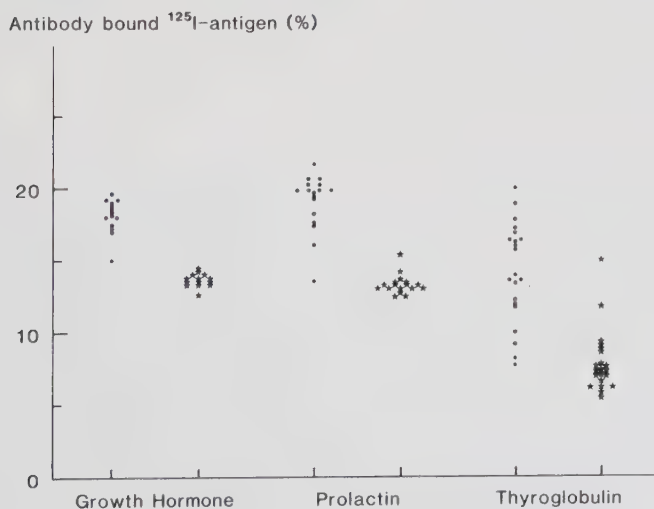
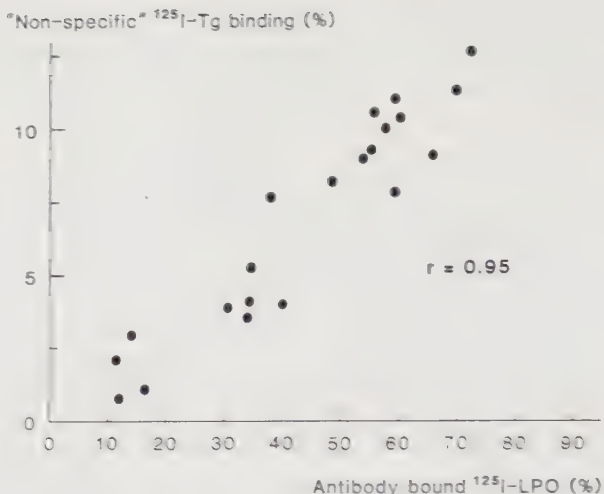


Fig. 2. The bound activity of the antigens iodinated with the lactoperoxidase method (●) and the chloramine-T method (*) as measured in the GH, prolactin and thyroglobulin antibody assays.

Fig. 3. Correlation between the antibody binding of ^{125}I -lactoperoxidase and the presumed non-specific binding, measured as the difference between the antibody binding of the ^{125}I -thyroglobulin iodinated with the lactoperoxidase and the chloramine-T methods, in the same serum.



(Fig. 4). On the same column, ^{125}I -LPO moves somewhat retarded from the Tg, but there is an obvious contamination with LPO in the fractions containing Tg. Similarly, ^{125}I -GH and ^{125}I -PrI, purified on Sephadex G-50 and Sephadex G-100 columns, respectively, are also contaminated with ^{125}I -LPO. The contaminating ^{125}I -LPO was markedly retarded and well separated from the GH and PrI when the tracer was purified with HPLC (data not shown).

As rabbit and goat serum are often used in radioimmunoassays, pooled sera from 10 different rabbits and serum samples from 2 different goats were tested with respect to the presence of antibodies to bovine LPO. None of these sera contained LPO antibodies.

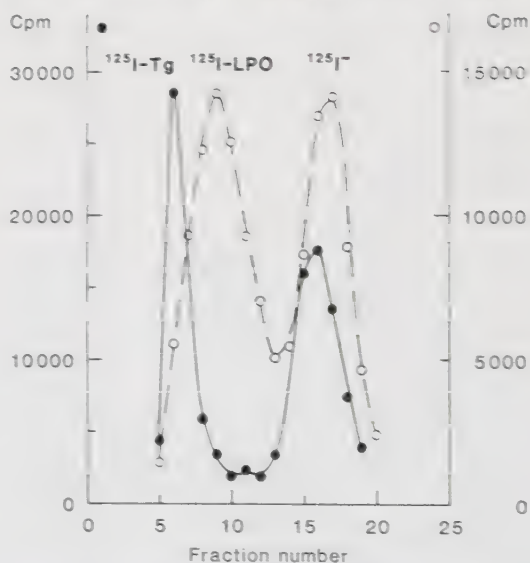


Fig. 4. Elution patterns of ^{125}I -thyroglobulin and ^{125}I -lactoperoxidase after chromatography on a Sephadex G-200 column (15 x 1 cm). The antigens were eluted with a barbital buffer, 0.075 mol/L, pH 8.6. Fractions of 1.0 ml were collected.

DISCUSSION

We have demonstrated that endogenous antibodies to LPO, an enzyme present in human milk as well as in cow milk, are present in the majority of healthy adults (89%), sometimes in high titres. Low concentrations of antibodies to other milk proteins have been found in a similarly high proportion of healthy individuals (7, 8). The occurrence of endogenous LPO antibodies may cause unforeseen interference in radioassays in which the antigen has been iodinated with the LPO method - a possible source of error that needs to be borne in mind when enzymatic iodination with LPO is used. LPO antibodies may interfere with the results in any radioassay system, in which the human IgG is precipitated with the antibodies, including radioassays in which human anti-IgG antibodies are used. In double antibody radioimmunoassays, where rabbit immunoglobulin is used as the precipitating agent, endogenous LPO antibodies will not affect the results.

The interference of LPO antibodies can be eliminated by using other purification procedures for the tracer, such as adsorption chromatography or high performance liquid chromatography, and by iodination with immobilized LPO or by iodination with chloramine-T.

REFERENCES

1. Thorell, J.I., and Johansson, B.G., Enzymatic iodination of polypeptides with ^{125}I to high specific activity. Biochim. Biophys. Acta 251, 363-369 (1971).
2. Thorell, J.I., and Larsson, I., Lactoperoxidase coupled to polyacrylamide for radio-iodination of proteins to high specific activity. Immunochemistry 11, 203-206 (1974).
3. Ericsson, U-B., Larsson, I., and Thorell, J.I., Purification and storage of thyroglobulin. Two important factors influencing the radioimmunoassay for thyroglobulin. In press. Scand. J. Clin. Lab. Invest.
4. Hunter, W.M., and Greenwood, F.C., Preparation of iodine-131 labelled human growth hormone of high specific activity. Nature 194, 495-496 (1962).

5. Thorell, J.I., and Larson, S.M., Radioimmunoassay and related techniques. Methodology and clinical applications. C.V. Mosby Co., St. Louis, 1978, pp 271-272.
6. Ericsson, U-B., Larsson, I., Murne, A., and Thorell, J.I., A new sensitive immunosorbent radioassay for the detection of circulating antibodies to polypeptide hormones and proteins. In press. Scand. J. Clin. Lab. Invest.
7. Savilahti, E., Immunochemical study of the malabsorption syndrome with cow's milk intolerance. Gut 14, 491-501 (1973).
8. Toivanen, A., Viljanen, M.K., and Savilahti, E., IgM and IgG anti-milk antibodies measured by radioimmunoassay in myocardial infarction. Lancet ii, 205-207 (1975).

SERUM THYROGLOBULIN IN DIFFERENTIATED THYROID CARCINOMA

U.-B. Ericsson, L. Tegler, S. Lennquist, S. Borup Christensen, E. Ståhl
and J. I. Thorell

*From the Departments of Nuclear Medicine, Internal Medicine and Surgery,
Malmö General Hospital, the Department of Oncology, University of Lund,
and the Departments of Internal Medicine and Surgery,
University of Linköping, Sweden*

(Submitted for publication May 16, 1983. Accepted January 10, 1984)

Abstract. Serum thyroglobulin (S-Tg) concentrations were measured in 262 patients with differentiated thyroid carcinoma. Analysis of S-Tg with respect to histology showed that the follicular variety of carcinoma had the highest S-Tg values preoperatively and postoperatively when metastases were found. The S-Tg levels during thyroxine treatment accurately predicted the results of ¹³¹I total body scan in all but two cases. In another patient both S-Tg and total body iodine scan were negative at the time when metastases were discovered at X-ray examination. All but two patients with negative scan had S-Tg <10 µg/l, whereas patients with metastases had values between 11 and >5000 µg/l. In patients with residual thyroid bed uptake the S-Tg range was from <2 to 84 µg/l. The authors conclude that determination of the S-Tg level can replace total body iodine scan in most patients who have undergone ablative therapy for differentiated thyroid carcinoma.

Elevated serum concentrations of thyroglobulin (S-Tg) may occur in patients with thyroid cancer (10, 11, 13, 18, 19, 21, 24), but also in non-neoplastic thyroid disorders (4, 6, 15). S-Tg thus does not selectively distinguish thyroid cancer in untreated patients. After ablative therapy, however, the S-Tg concentration may serve as a marker for residual or recurrent thyroid cancer (3, 7, 12, 14, 17, 19, 20, 24).

Total body iodine scan (TBS) is used for diagnosing functioning local recurrences and metastases in thyroid carcinoma patients. Comparisons of sensitivity and specificity between TBS and S-Tg have given conflicting results (1, 2, 3, 5). Possible reasons for the discrepancies include variations in the tumour morphology (and its typing), in completeness of the ablative therapy, and in the scanning performance, depending on e.g. the dose of ¹³¹I-iodide and the quality of the scan.

Our aim was to evaluate the clinical usefulness of S-Tg determination in relation to ¹³¹I-iodine scan in the routine follow-up of thyroid carcinoma patients after ablative therapy, and thereby to assess the possibility that S-Tg analysis can replace TBS in this situation.

MATERIAL AND METHODS

Patients

The study comprised 262 patients with differentiated thyroid carcinoma (70 men and 192 women collected from 3 thyroid centres). S-Tg was measured before treatment in 82 patients (indicated below by figures in brackets) and after treatment in 240. The diagnosis was confirmed by histologic examination of the gland after thyroidectomy in all patients. The tumours were typed in accordance with the WHO classification (8). As it has been proposed that Hürthle cell adenomas do not synthesize Tg (9), the Hürthle cell carcinomas were studied separately.

Follicular carcinoma was present in 106, (32) patients, and was classified as Hürthle cell carcinoma in 15 (6) of them. Papillary carcinoma was the classification in 135 (37) patients and medullary carcinoma in 21 (13). The follow-up periods and clinical data are presented in Table I. Patients with metastases, and their S-Tg levels, are listed in Table II.

The postoperative follow-up procedure varied between the three centres. All 262 patients were clinically evaluated. In 224 of them an initial postoperative TBS was performed after a period without thyroid replacement therapy. In 41 patients S-Tg was analyzed just before a follow-up TBS one year or more after the initial treatment. All other samples were taken during thyroxine treatment. The postoperative substitution therapy was given in a dosage which kept the basal TSH concentration at a low normal level, <3 mIU/l.

The half-life of circulating Tg in man is variable (25). Accordingly, the time until a stable postoperative Tg value is obtained will vary from a few weeks to several months. For this reason, only S-Tg values obtained six months or more after surgery were included in the study.

Table I. Clinical data in 286 patients with thyroid carcinoma

Tg ab = thyroglobulin antibody

Histologic type	No. of patients	Age, yrs: median (range)	Postoperative observation time, months: median (range)	Tg ab ^a positive	Operation ^a				
					Total thyroid-ectomy	Hemi-thyroid-ectomy	Subtotal thyroid resection	131-I therapy	External irradiation
Follicular	91	58 (18-86)	44 (7-258)	4	62	19	8	55	11
Hürthle cell	15	65 (38-77)	36 (15-106)	0	7	8	0	4	1
Papillary	135	52 (15-83)	51 (7-307)	16	93	30	12	52	19
Medullary	21	61 (17-80)	60 (8-276)	1	20	0	1	3	1

^a In one of the study centres (Malmö) operations less radical than total thyroidectomy were sometimes performed during the observation period.

Controls

The controls were 400 women, aged 48 and 53 years, who were investigated as part of a population screening programme. Thyroid disease, current or previous, was excluded by thorough clinical and laboratory investigation.

Thyroglobulin assay

S-Tg was measured with a double antibody radioimmuno-logic assay using, with slight modifications, the technique described by van Herle and co-workers (23). Purified Tg, used to raise the first antibody, was kindly provided by Dr van Herle (University of California, Los Angeles, USA). The minimum detectable level was 2 µg/l. The intra-assay coefficient of variation, estimated from duplicate determinations of samples with S-Tg concentrations between 10-200 µg/l, was 6.3%. The reproducibility was ascertained by inclusion of three different control sera in all assays. The results from the measurements of these sera over one year were 0.8 ± 0.7 µg/l, 9.4 ± 1.5 µg/l and 29.5 ± 4.1 µg/l (mean $\pm$ SD).

All samples were tested for presence of antithyroglobulin antibodies with a passive haemagglutination test, using a commercial kit (Thymune-T, Wellcome Research Laboratories, Beckenham, England). Positive results were recorded when the dilution was higher than 1:10. Patients with a positive test were included, but are reported separately.

Statistics

For statistical analyses we used the Mann-Whitney U-test for unpaired observations. S-Tg values less than 2 µg/l were assigned a value of 1 µg/l. Those above 5000 µg/l were assigned a value of 6000 µg/l.

RESULTS

Controls

The S-Tg concentrations in the 400 healthy women ranged from nondetectable to 77 µg/l, with a median

value of 13 µg/l and the 97.5th percentile equal to 40 µg/l. This reference range was used for all patients but the thyroidectomized. A tentative reference range was established from all thyroidectomized patients without metastases or uptake on TBS, and with no demonstrable thyroglobulin antibodies ($n=86$). The median value was 3 µg/l and the 97.5th percentile was 10 µg/l. This was taken as the upper reference limit after total thyroidectomy.

Follicular carcinoma (Fig. 1)

Before treatment. 26 patients were studied (median S-Tg 155 µg/l, range 28->5000 µg/l). The S-Tg level was elevated in 21 of these patients. The distribution range of the S-Tg values was the same in the five patients with metastases as in the 21 without evidence of secondary tumour. Of the five patients with metastases, one had normal S-Tg level and follicular carcinoma of low differentiation.

After treatment, all 14 patients with metastases had S-Tg >10 µg/l. Most of the patients with the highest values (>5000 µg/l) had bone metastases. Of the 70 patients without metastases, 61 had S-Tg $\leq$ 10 µg/l. Seven of the nine with a S-Tg concentration above the reference limit had residual thyroid bed tissue on TBS, whereas two had no uptake on scan, negative chest X-ray and no clinical evidence of metastases. There was no variation in the S-Tg pattern after surgery, irrespective of the interval between therapy and sampling, unless metastases developed.

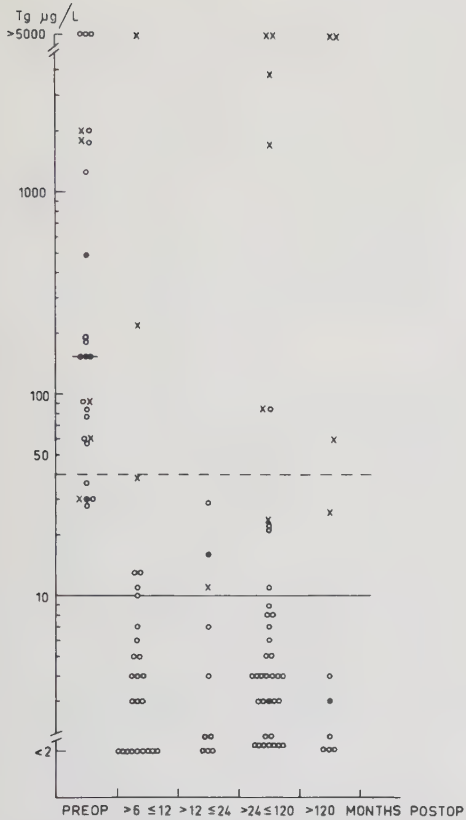


Fig. 1. Preoperative and postoperative S-Tg values in patients with follicular carcinoma. The interrupted horizontal line represents the upper limit of the normal range. The unbroken horizontal line represents the upper limit of the normal range after total thyroidectomy. The short solid line represents the median value. ○ = patients without Tg antibodies, ● = patients with Tg antibodies, × = patients with metastases.

Hürthle cell carcinoma (Fig. 2)

Before treatment, six patients were studied (median S-Tg 70 µg/l, range 23–670 µg/l). S-Tg was elevated in five of these patients. The median S-Tg concentration was significantly higher than in the papillary carcinoma group ($p < 0.05$), but did not differ from that in the follicular carcinoma group ($p > 0.05$).

After treatment, all three patients with metastases had elevated S-Tg values. One patient without metastases, but with residual thyroid bed tissue, had a S-Tg concentration equal to 15 µg/l. In all the

Table II. S-Tg in patients with differentiated thyroid carcinoma with metastases (50/262), according to type and extent of the tumour

Tg ab = thyroglobulin antibody

Histology	Metastases	S-Tg (µg/l)	Tg ab
<i>Untreated group</i>			
Follicular	Lymph node	61	—
Follicular	Lymph node, local	91	—
Follicular	Liver, bone	2 000	—
Follicular	Liver, bone	30	+
Follicular	Bone	1 800	—
Hürthle cell	Lymph node, lung	23	—
Papillary	Lymph node	7	—
Papillary	Lymph node	37	—
Papillary	Lymph node	16	—
Papillary	Lymph node	32	—
Papillary	Lymph node	11	—
Papillary	Lymph node	91	—
Papillary	Lymph node	21	—
Papillary	Lymph node	77	—
Papillary	Lymph node	50	—
Papillary	Lymph node	15	—
Papillary	Lymph node	48	—
Papillary	Lymph node	42	—
Papillary	Lymph node	450	—
Papillary	Lymph node	29	—
Papillary	Lymph node, local	1 200	—
Medullary	Lymph node	29	—
Medullary	Lymph node	95	—
Medullary	Lymph node	27	—
Medullary	Widespread	25	—
<i>Treated group</i>			
Follicular	Lung	38	—
Follicular	Lung	60	—
Follicular	Lung	84	—
Follicular	Lymph node, lung	26	—
Follicular	Liver	6 100	—
Follicular	Bone	11	—
Follicular	Bone	24	—
Follicular	Bone	224	—
Follicular	Bone	1 750	—
Follicular	Bone	3 900	—
Follicular	Lymph node, bone	7 000	—
Follicular	Lymph node, bone	51 800	—
Follicular	Lung, bone	14 000	—
Follicular	Local, lung, bone	7 700	—
Hürthle cell	Lung	110	—
Hürthle cell	Lymph node, lung	370	—
Hürthle cell	Lymph node, lung	147	—
Papillary	Lung	24	—
Papillary	Lymph node, lung	13	—
Papillary	Lymph node, lung	18	—
Papillary	Lymph node, local, lung	245	—
Papillary	Lymph node, bone	1 600	—
Medullary	Local	<2	—
Medullary	Lung	8	—
Medullary	Widespread	2	—

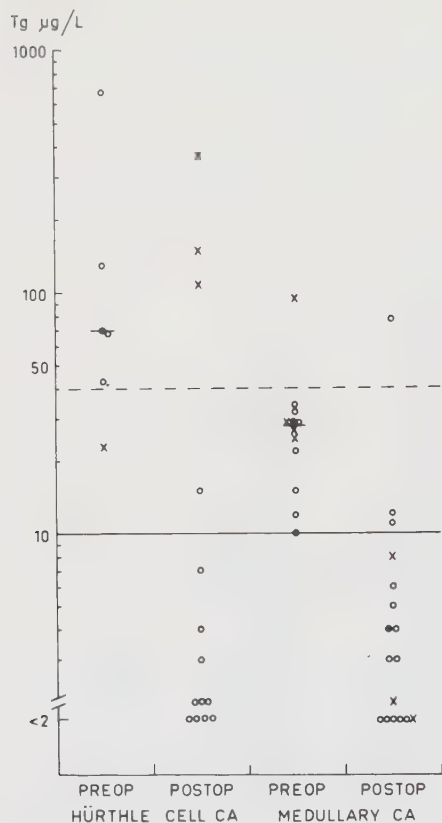


Fig. 2. Preoperative and postoperative S-Tg values in patients with Hürthle cell carcinoma or medullary carcinoma. The interrupted horizontal line represents the upper limit of the normal range. The unbroken horizontal line represents the upper limit of the normal range after total thyroidectomy. The short solid lines represent the median values. ○ = patients without Tg antibodies, ● = patients with Tg antibodies, × = patients with metastases.

other patients without metastases the S-Tg concentration was <10 µg/l.

Papillary carcinoma (Fig. 3).

Before treatment, 37 patients were studied (median S-Tg 37 µg/l, range 7–1200 µg/l). Raised S-Tg concentration was found in 16 of these patients, the values in patients with metastases being similar to those without secondary tumours ($p>0.1$). The median S-Tg for papillary carcinomas was lower than

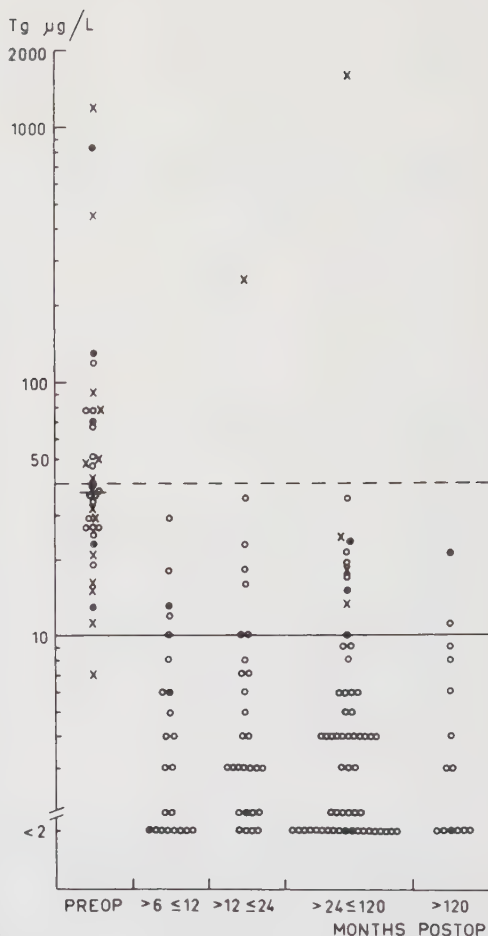


Fig. 3. Preoperative and postoperative S-Tg values in patients with papillary carcinoma. The interrupted horizontal line represents the upper limit of the normal range. The unbroken horizontal line represents the upper limit of the normal range after total thyroidectomy. The short solid line represents the median value. ○ = patients without Tg antibodies, ● = patients with Tg antibodies, × = patients with metastases.

that for follicular carcinomas ($p<0.001$). The patient with the highest S-Tg value (1200 µg/l) had extensive local metastases, which on histologic examination was found partly to consist of follicular formations with exuberant epithelial cells. Of the other 14 patients with lymph-node metastases, eight had normal S-Tg levels.

After treatment, all five patients with distant metastases had elevated S-Tg values. One patient with pulmonary metastases, which did not accumulate radioiodine, had a S-Tg of 6 $\mu\text{g/l}$ at the time when these metastases were discovered on chest X-ray. Not until two years after that date did her S-Tg rise above 10 $\mu\text{g/l}$ on thyroxine treatment. Of the 120 patients without metastases, 104 had S-Tg values within the reference range. All 16 patients without metastases but with S-Tg higher than 10 $\mu\text{g/l}$ had residual thyroid bed tissue. As in follicular carcinoma, the S-Tg pattern did not change after surgery unless metastases developed.

Medullary carcinoma (Fig. 2)

Before treatment, 13 patients were studied (median S-Tg 28 $\mu\text{g/l}$, range 10–95 $\mu\text{g/l}$). One of them had an elevated S-Tg level. In three of the four patients with metastases the S-Tg was within the normal range. The median S-Tg value was lower than in papillary and in follicular carcinoma (both groups $p < 0.001$).

After treatment, all three patients with metastases had S-Tg below 10 $\mu\text{g/l}$. In the patient with the highest S-Tg concentration, but without metastases, a subtotal thyroid resection had been performed because of thyrotoxicosis and the cancer was an incidental finding. In the two other patients with slightly elevated S-Tg, no postoperative TBS was performed.

Comparison between postoperative S-Tg and type of surgery and substitution therapy

The range of the S-Tg values was wider in patients with postoperative residual thyroid tissue than in those without detectable neck uptake on TBS, and the respective median values differed significantly (4 $\mu\text{g/l}$ and 3 $\mu\text{g/l}$, $p < 0.001$). Of 101 patients with residual thyroid bed tissue, 19 had values $> 10 \mu\text{g/l}$, but only one patient with negative TBS had such a value. All the patients with metastases (medullary carcinoma not included) had S-Tg $> 10 \mu\text{g/l}$ (Fig. 4).

The range of the postoperative S-Tg values was the same in patients without metastases who had undergone hemithyroidectomy ($n = 78$) and total thyroidectomy ($n = 182$), although the median values differed significantly (6 $\mu\text{g/l}$ and 4 $\mu\text{g/l}$, $p < 0.001$). These values are shown in Fig. 5.

The postoperative S-Tg concentrations approximately doubled when thyroxine was discontinued in six patients with residual thyroid tissue, but of

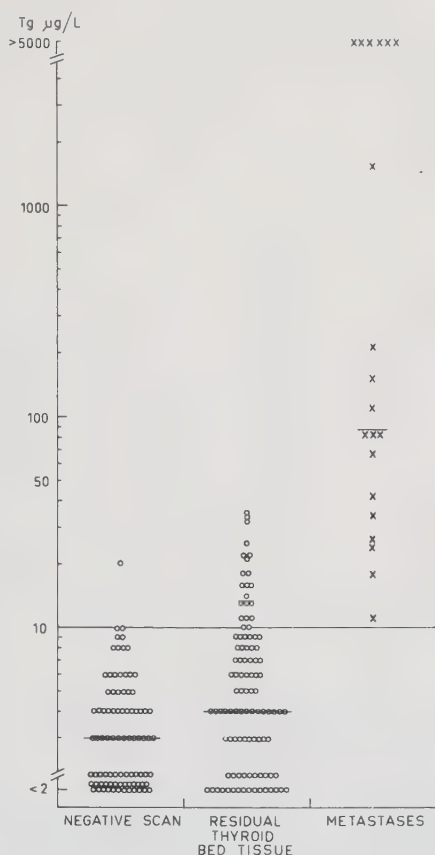


Fig. 4. S-Tg concentrations in patients with negative scan, with residual thyroid bed tissue, and with metastases. The upper limit of the normal range after total thyroidectomy is indicated by the unbroken horizontal line. The short solid lines represent the median values.

the eight patients with negative TBS all but three showed much less change in this respect (Fig. 6).

Comparison between S-Tg concentration and total body scan uptake

Only two patients showed discrepancy between result of S-Tg determination and of TBS. Both had negative TBS but elevated S-Tg levels with as well as without T_4 treatment. No metastases were found in thorough clinical and X-ray observation for six years. In addition, one patient had normal S-Tg and negative scan at the time when pulmonary metastases were roentgenographically detected. Two years

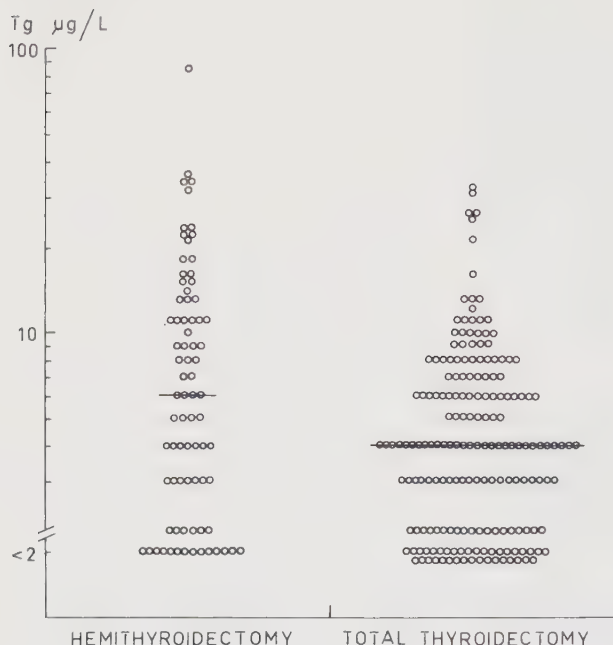


Fig. 5. Postoperative S-Tg concentrations during thyroxine substitution therapy in patients without metastases after hemithyroidectomy or total thyroidectomy. The short solid lines represent the median values.

later her S-Tg level rose to 18 µg/l while TBS remained negative.

Influence of thyroglobulin antibodies

No correlation was found between the prevailing Tg antibody titre and the S-Tg concentration (Fig. 7). As Fig. 8 illustrates, a gradual decrease in the S-Tg level could be observed postoperatively despite the presence of Tg antibodies. The S-Tg values fell to one-sixth of the preoperative value although the antibody titre remained elevated.

DISCUSSION

The value of S-Tg measurements in relation to TBS in follow-up after ablative therapy for differentiated thyroid carcinoma has been disputed. As recent authors have pointed out (1), the reason is that a variety of factors may influence results of the determinations.

An important factor which has not been taken into account in this connection is the histologic classification of the tumours. The classification may differ from study to study, and often it is not

stated. Some pathologists use the WHO classification (8), in which all tumours with papillary structures are referred to as papillary carcinomas, regardless of the occurrence of follicular and solid areas. Other schemes distinguish between "pure" papillary and mixed papillary-follicular structures. However, individual papillary carcinomas and mixed carcinomas may vary markedly in the amount of follicular areas they are found to contain. This variation may explain part of the differences in S-Tg concentrations that have been observed among patients with papillary carcinoma.

Even after total thyroidectomy, remnants of normal thyroid tissue capable of secreting Tg may remain. This decreases the diagnostic sensitivity of S-Tg determinations (17). The amount of thyroid cells that are required to secrete a measurable amount of Tg to the circulation is not known. Our own results indicate that remnants too small to be detected by TBS may cause readily detectable serum concentrations, though these as a rule are less than 10 µg/l. The sensitivity of TBS permits identification of patients with residual thyroid tissue which can raise the S-Tg level sometimes to a degree

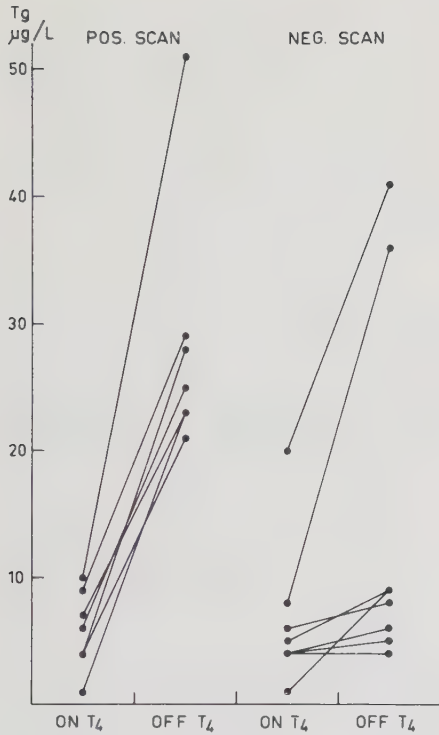


Fig. 6. S-Tg concentrations during and without thyroid replacement therapy in patients with positive and with negative scan.

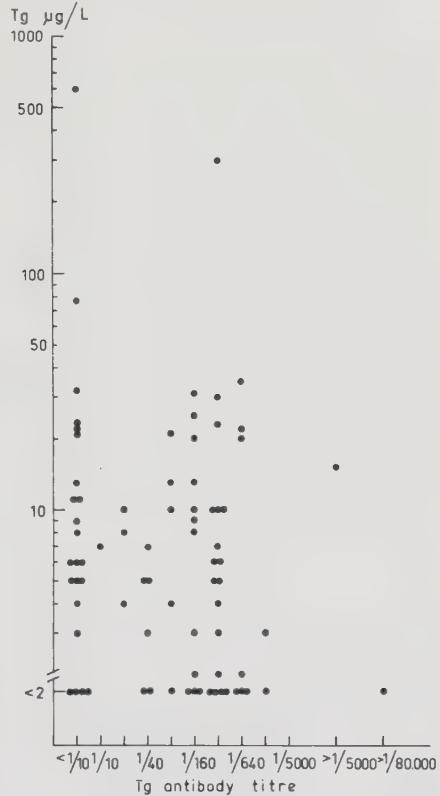


Fig. 7. Postoperative S-Tg concentration and Tg antibody titre patients with differentiated thyroid carcinoma.

comparable with metastatic thyroid tissue. Postoperative TBS will therefore enhance the accuracy of postoperative S-Tg evaluation. Complete thyroidectomy or, if required, radioablation of residual thyroid tissue will increase the specificity of postoperative S-Tg determination.

TSH has been shown to increase the Tg release from normal thyroid (22) and also from neoplastic thyroid tissue even when the tumour has no capacity to accumulate radioiodine (16). The extent of the rise in S-Tg concentration seen during the hypothyroid period depends not only on the amount of thyroid tissue and the capacity to produce Tg, but also on the prevailing TSH concentration. If S-Tg is measured during hypothyroidism, there will be an intraindividual variation which depends on that TSH concentration. Thus, small rises indicative of metastasis can be obscured by fluctuations induced

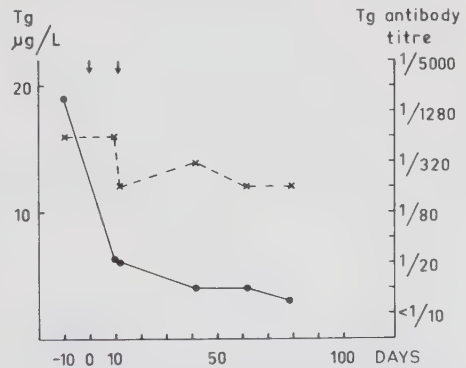


Fig. 8. The postoperative change in S-Tg concentration and the Tg antibody titre in a patient with differentiated papillary carcinoma. Arrows indicate hemithyroidectomy. ●—●, S-Tg values; ×—×, Tg antibody titre.

by changing stimulation from the variable TSH concentration. S-Tg results obtained during the hypothyroid situation consequently are difficult to evaluate. S-Tg preferably should be measured at a stable TSH concentration, which is most easily achieved during thyroxine suppression.

In this study three patients with metastases had "normal" S-Tg levels postoperatively. All three had medullary carcinoma. Of 27 patients with postoperative S-Tg concentration higher than $10 \mu\text{g/l}$, 25 had detectable residual uptake in the neck at TBS. Whether the observed rise in S-Tg in the two patients with negative TBS and no other signs of metastases represented false positive results cannot yet be judged, because of the sometimes very slow growth rate of secondary thyroid tumours. But if the results prove to be indicative of tumour, determination of S-Tg can be assumed to be a more sensitive method than TBS for early detection of local recurrence or metastasis.

CONCLUSIONS

The study demonstrated that S-Tg measurements are valuable in follow-up after total thyroidectomy for differentiated thyroid carcinoma. The method correlates well with scanning results when performed during thyroxine treatment. In some cases it permits detection of nonfunctioning metastases. S-Tg measurement has the additional advantage of reducing the total radioactive dose given to the patient, and the possibility of examining the patients during substitution therapy reduces the morbidity from induced hypothyroidism. Further, the potential risk of stimulating residual thyroid cancer cells with TSH during the hypothyroid period is avoided. S-Tg measurements, making much smaller demands on personnel, laboratories, etc., optimize cost-effectiveness in management of the patient. In our view, therefore, S-Tg measurement must be regarded as a routine procedure in the follow-up of differentiated thyroid carcinoma after total thyroidectomy.

REFERENCES

1. Ashcraft, M. W. & van Herle, A. J. 1981. The comparative value of serum thyroglobulin measurements and iodine 131 total body scans in the follow-up study of patients with treated differentiated thyroid cancer. *Am J Med* 71, 806.
2. Castagnoli, A., Forni, S., Milani, S., Pupi, A. & Cappelli, G. 1982. Pitfalls of serum thyroglobulin assay in the follow-up of differentiated cancer of the thyroid. In *Nuclear medicine and biology. Proceedings of the 3rd World Congress of Nuclear Medicine and Biology (Paris, 1982)*, part IV, p. 2791. Pergamon Press.
3. Charles, M. A., Dodson, L. E., Waldeck, N., Hofeldt, F., Ghaed, N., Telepak, R., Ownbey, J. & Burstein, P. 1980. Serum thyroglobulin levels predict total body iodine scan findings in patients with treated well-differentiated thyroid carcinoma. *Am J Med* 69, 401.
4. Eber, M., Abecassis, J., Grob, J. C., Ott, G. & Methelin, G. 1980. Immunoradiometric assay for human thyroglobulin and variations in thyroid pathology. *Clin Chim Acta* 105, 51.
5. Echenique, R. L., Kasi, L., Haynie, T. P., Glenn, H. J., Samaan, N. A. & Hill, C. S. 1982. Critical evaluation of serum thyroglobulin levels and I-131 scans in post-therapy patients with differentiated thyroid carcinoma: concise communication. *J Nucl Med* 23, 235.
6. Gardner, D. F., Rothman, J. & Utiger, R. D. 1979. Serum thyroglobulin in normal subjects and patients with hyperthyroidism due to Graves' disease: Effects of T_3 , iodide, ^{131}I and antithyroid drugs. *Clin Endocrinol* 11, 585.
7. Hagemann, J. & Schneider, C. 1977. Control of treatment of differentiated thyroid carcinoma by measurement of thyroglobulin in serum. In *IAEA. International Symposium on Radioimmunoassay and Related Procedures in Medicine (West Berlin, 1977)*, p. 363. IAEA-SM-220/23.
8. Hedinger, C. & Sobin, L. H. WHO 1974. Histological typing of thyroid tumours. *International histological classification of tumours*, No. 11, Geneva.
9. Heimann, P., Ljunggren, J.-G., Löwhagen, T. & Hjern, B. 1973. Oxyphilic adenoma of the human thyroid. A morphological and biochemical study. *Cancer* 31, 246.
10. Hjort, T. 1963. "Thyroglobulin" in the serum. *Acta Med Scand* 174, 137.
11. Lo Gerfo, P., Colacchio, D., Stillman, T. & Feind, C. 1977. Serum thyroglobulin and recurrent thyroid cancer. *Lancet* i, 881.
12. McDougall, I. R. & Bayer, M. F. 1980. Follow-up of patients with differentiated thyroid cancer using serum thyroglobulin measured by an immunoradiometric assay. Comparison with I-131 total body scans. *J Nucl Med* 21, 741.
13. Pacini, F., Pinchera, A., Giani, C., Grasso, L., Doveri, F. & Baschieri, L. 1980. Serum thyroglobulin in thyroid carcinoma and other thyroid disorders. *J Endocrinol Invest* 3, 283.
14. Pacini, F., Pinchera, A., Giani, C., Grasso, L. & Baschieri, L. 1980. Serum thyroglobulin concentrations and ^{131}I whole body scans in the diagnosis of metastases from differentiated thyroid carcinoma (after thyroidectomy). *Clin Endocrinol* 13, 107.
15. Pezzino, V., Cozzani, P., Filetti, S., Galbiati, A., Lisi, E., Squatrito, S. & Vigneri, R. 1977. A radioimmunoassay for human thyroglobulin: methodology and clinical applications. *Eur J Clin Invest* 7, 503.
16. Schlumberger, M., Charbord, P., Fragu, P., Lumbroso, J., Parmentier, C. & Tubiana, M. 1980. Circu-

- lating thyroglobulin and thyroid hormones in patients with metastases of differentiated thyroid carcinoma: Relationship to serum thyrotropin levels. *J Clin Endocrinol Metab* 51, 513.
17. Schlumberger, M., Fragu, P., Parmentier, C. & Tubiana, M. 1981. Thyroglobulin assay in the follow-up of patients with differentiated thyroid carcinomas: comparison of its value in patients with or without normal residual tissue. *Acta Endocrinol* 98, 215.
18. Shlossberg, A. H., Jacobson, J. C. & Ibbertson, H. K. 1979. Serum thyroglobulin in the diagnosis and management of thyroid carcinoma. *Clin Endocrinol* 10, 17.
19. Shah, D. H., Dandekar, S. R., Jeevenram, R. K., Kumar, A., Sharma, S. M. & Ganatra, R. D. 1981. Serum thyroglobulin in differentiated thyroid carcinoma: histological and metastatic classification. *Acta Endocrinol* 98, 222.
20. Tang Fui, S. C. N. G., Hoffenberg, R., Maisey, M. N. & Black, E. G. 1979. Serum thyroglobulin concentrations and whole-body radioiodine scan in follow-up of differentiated thyroid cancer after thyroid ablation. *Br Med J* 2, 298.
21. Torrigiani, G., Doniach, D. & Roitt, I. M. 1969. Serum thyroglobulin levels in healthy subjects and in patients with thyroid disease. *J Clin Endocrinol Metab* 29, 305.
22. Uller, R. P., van Herle, A. J. & Chopra, I. J. 1973. Comparison of alterations in circulating thyroglobulin, triiodothyronine and thyroxine in response to exogenous (bovine) and endogenous (human) thyrotropin. *J Clin Endocrinol Metab* 37, 741.
23. van Herle, A. J., Uller, R. P., Matthews, N. L. & Brown, J. 1973. Radioimmunoassay for measurement of thyroglobulin in human serum. *J Clin Invest* 52, 1320.
24. van Herle, A. J. & Uller, R. P. 1975. Elevated serum thyroglobulin. A marker of metastases in differentiated thyroid carcinomas. *J Clin Invest* 56, 272.
25. van Herle, A. J., Vassart G. & Dumont, J. E. 1979. Control of thyroglobulin synthesis and secretion. *N Engl J Med* 301, 307.

A Compound Follicular-Parafollicular Cell Carcinoma of the Thyroid: A New Tumor Entity?

OTTO LJUNGBERG, MD,* ULLA-BRITT ERICSSON, MD,† LENNART BONDESON, MD,*
AND JAN THORELL, MD†

An unusual thyroid carcinoma is described, showing structural, histochemical and radioimmunologic features of both a follicular and a parafollicular cell carcinoma. Radioimmunoassay revealed high levels of thyroglobulin in the patient's serum and in extracts from metastatic tumor tissue. Immunoreactive thyroglobulin was demonstrated histochemically in tumor cells. On scanning, pulmonary metastases showed uptake of ^{131}I . Somatostatin and neurotensin immunoreactivity was also revealed histochemically in the tumor and a large proportion of the neoplastic cells were argyrophil. Serum calcitonin level was normal and no immunoreactive calcitonin was found in tumor tissue by radioimmunoassay or histochemically. Light microscopy showed cribriform patterns suggestive of follicular carcinoma as well as solid areas reminiscent of medullary carcinoma. Electron microscopy revealed two types of tumor cells. One type had electron dense granules resembling secretory granules characteristic of polypeptide hormone and/or monoamine producing endocrine cells. The other type had no such granules but showed a prominent vesicular rough endoplasmic reticulum similar to that seen in neoplastic follicular cells. The results suggest two alternative possibilities regarding the histogenesis of the tumor. One would be a mixed neoplasm, resulting from a coincidental malignant change in both follicular and parafollicular thyroid cells. The other, more likely alternative would be that the tumor cells are derived from a common stem cell with the potentiality of differentiating into both follicular and parafollicular adult cells. The finding that both thyroglobulin and somatostatin or neurotensin immunoreactivity occurred together in some tumor cells supports the latter possibility and suggests that at least some follicular and parafollicular cells may have a common precursor origin.

Cancer 52:1053-1061, 1983.

It is generally believed that differentiated thyroid carcinoma can be divided into two histogenetically distinct categories, *i.e.*, follicular and papillary carcinoma which are derived from the follicular cells, associated with the production of thyroglobulin and thyroid hormones; and medullary carcinoma, which is derived from the parafollicular cells (C-cells), associated with the production of calcitonin and other hormonal peptides.

Medullary carcinoma of the thyroid is defined as a neoplasm which is capable of producing calcitonin and may contain a characteristic amyloid stroma and argyrophil tumor cells having intracytoplasmic secretory granules of endocrine type.¹ Besides calcitonin, this tumor may also elaborate other substances, including peptide hormones such as ACTH,² somatostatin^{3,4} and sub-

stance P,⁵ as well as certain monoamines, such as catecholamines and 5-hydroxytryptamine.⁶⁻⁸ So far, none of these features have been reported to occur in follicular or papillary thyroid carcinoma.

Nevertheless, we report here a case of thyroid carcinoma with structural, histochemical and radioimmunologic features of both of the aforementioned tumor categories, suggesting that a clear distinction between the two may not always be as valid as previously believed.

Case Report

A 45-year-old woman, without a family history of thyroid disease, noted a rapidly growing swelling in the neck 2 months prior to admission. The physical examination revealed a hard tumor in the left thyroid lobe. The right lobe and isthmus were diffusely enlarged. Otherwise the clinical investigations and routine biochemical studies (including serum calcium) were unremarkable. The thyroid hormone values, including total thyroxine and triiodothyronine in serum, and free thyroxine- and triiodothyronine-index, were low normal. Serum thyroglobulin and calcitonin measurements were not done on this

Presented in part at the VIIIth European Congress of Pathology, Helsinki, Finland, August 30-September 4, 1981.

From the Departments of Pathology,* and Nuclear Medicine,† University of Lund, Malmö General Hospital, Malmö, Sweden.

Address for reprints: Otto Ljungberg, MD, Department of Pathology, University of Lund, Malmö General Hospital, S-21401 Malmö, Sweden.

Accepted for publication June 14, 1982.

occasion. The radiographic examination of lungs and bone showed normal results. The thyroid scan revealed a cold nodule in the lower part of the left lobe. The uptake in the rest of the gland was irregular.

A total thyroidectomy was performed two months after presentation. The left lobe showed an ill-defined tumor, $4 \times 4.5 \times 6$ cm, which had invaded the extrathyroidal tissue and involved lymph nodes close to the gland. The cut surfaces of the tumor showed a greyish-yellow, finely granular pattern with irregular necrotic areas. The primary pathologic diagnosis was an unusual thyroid carcinoma; because it contained argyrophil tumor cells it was considered as probably related to medullary carcinoma. The patient was given postoperative replacement therapy but no radiotherapy.

Because of the tentative diagnosis of a medullary carcinoma a thorough endocrine evaluation was performed three months after the operation. This included the following determinations; serum PTH, serum calcitonin measured basally and after whisky and calcium stimulation,⁹ serum gastrin measured basally and after whisky stimulation, thyroid hormones in serum and urinary excretion of noradrenaline and adrenaline. The results were quite normal but for a markedly elevated serum thyroglobulin level, $>4000 \mu\text{g/l}$ (normal value, $<40 \mu\text{g/l}$). A ^{131}I scan showed a distinct uptake at the larynx level which was thought to be an activated pyramidal lobe.

Seven months after the thyroid operation, a large tumor mass behind the left clavicle was extirpated and found to represent large lymph node metastases. The serum thyroglobulin level fell to $51 \mu\text{g/l}$ postoperatively. During the following 3 years, the course was uneventful, except that the serum thyroglobulin level raised slowly, reaching $180 \mu\text{g/l}$ after three years. Repeated measurements of serum calcitonin invariably showed normal values.

Four years after the first operation, the patient developed a left-sided pleurisy and bilateral pulmonary metastases were diagnosed radiographically. A ^{131}I whole body scan was performed after a temporary interruption of the thyroid medication. Besides a small uptake corresponding to the pyramidal lobe, it also revealed uptake in some of the pulmonary metastases. The patient was treated with an eradication dose of ^{131}I , followed by 5-fluorouracil and Adriamycin. Her clinical condition improved temporarily. One year later however, she developed a hemiparesis and a parietal brain metastasis was removed. Her condition gradually deteriorated and she died after 3 months.

At necropsy there were extensive metastases in the mediastinum with infiltration of the tumor into the pericardium and the heart. Metastases were present in both lungs, the largest deposits being situated in those parts which had shown an ^{131}I uptake on scanning. Secondary tumor deposits were also found in the liver and kidneys.

Materials and Methods

Light Microscopy and Histochemistry

Specimens from the thyroid tumor and cervical lymph node metastases were fixed in 10% buffered formalin and embedded in paraffin. Sections for light mi-

croscopy were stained with hematoxylin and eosin. The alkaline Congo red technique¹⁰ was used for the evaluation of amyloid deposits. Masson-Hamperl's method¹¹ and the diazo-coupling technique¹² were applied for the demonstration of argentaffin material and 5-hydroxytryptamine respectively. Argyrophilia was visualized with Grimelius' silver nitrate procedure,¹³ using sections which had been refixed in Bouin's solution for 3 hours.

Immunolocalization Techniques

Sections were deparaffinized, hydrated and thoroughly washed in buffer overnight. The indirect immunofluorescence method (IF)¹⁴ and the peroxidase-antiperoxidase technique (PAP)¹⁵ were employed. The sections were incubated overnight at $+4^\circ\text{C}$ with the following antisera: two different rabbit anti-human calcitonin sera (1. I. MacIntyre, Endocrine Unit, Royal Postgraduate Medical School, London, England; dilutions IF 1/50, PAP 1/300 and 2. Immuno Nuclear Cooperation, Stillwater, Minnesota, USA; dilution PAP 1/500), rabbit antihuman somatostatin serum (J. F. Rehfeld, Rigshospitalet, Copenhagen, Denmark; dilutions IF 1/50, PAP 1/3000), two different rabbit antihuman neurotensin sera (1. N. S. Track, University of Toronto, Canada; dilutions IF 1/50, PAP 1/800 and 2. MILAB, Box 20047, Malmö, Sweden; dilution PAP 1/5000), and rabbit antihuman thyroglobulin serum (MILAB, Box 20047, Malmö, Sweden; dilutions IF 1/2000, PAP 1/10,000).

Controls: Sections from the following human tissues were used as positive controls: medullary thyroid carcinoma with amyloid stroma and known calcitonin production (for calcitonin), normal pancreatic tissue (for somatostatin), normal lower ileum (for neurotensin) and normal thyroid tissue (for thyroglobulin). Negative controls were performed by incubation of some sections with antiserum which had been inactivated by excess amount of the homologous hormone ($10\text{--}200 \mu\text{g}$ added to 1 ml of the diluted antiserum) and by incubation of other sections with nonimmune rabbit serum instead of the specific rabbit antihuman serum.

Cross absorption studies: To determine the possibility of cross-reaction between antibodies to thyroglobulin and the peptide hormones, sections were also incubated with rabbit antihuman thyroglobulin serum to which had been added calcitonin, somatostatin or neurotensin, respectively (same concentrations as for the negative controls).

Ten cases of differentiated thyroid carcinoma which were strictly follicular or papillary in type were included as additional controls to investigate whether these tumors contained antigens which cross-reacted with the antisera to calcitonin, somatostatin or neurotensin. Fur-

thermore, 20 cases of classical sporadic and familial medullary thyroid carcinoma, all with known calcitonin production, were also studied with the antihuman thyroglobulin serum to determine if this group of tumors contained antigens which cross-reacted with antibodies to thyroglobulin.

Double immunostaining: Double staining of sections from the tumor was carried out to determine whether immunoreactivity for thyroglobulin and for either of the neuropeptides studied, occurred in the same cells. The IF method was employed first to demonstrate either of the peptide hormones and an area of the section with tumor cells showing specific fluorescence was photographed. Then the antibodies of the first layer were removed as described by Tramu *et al.*¹⁶ by treating the section for 60 seconds with a solution of 0.15 M KMnO_4 and 0.01 N H_2SO_4 (pH 1.8). Removal of the antibodies was checked by reincubating the section with the fluorescent second layer, *i.e.* FITC-labeled sheep antirabbit serum and verifying the disappearance of specific fluorescence in the fluorescence microscope. After rinsing in buffer, the section was then processed according to the PAP-method using antibodies to thyroglobulin. The relevant area of the section was again found in the light microscope and rephotographed.

Electron Microscopy

Only paraffin blocks with formalin-fixed tumor tissue were available. Blocks measuring about 1 mm³ were cut out from the paraffin embedded tumor specimens. The tissue was hydrated, refixed in 1% OsO_4 in 0.1 M phosphate buffer pH 7.4, treated with uranyl acetate and phosphotungstic acid, dehydrated and embedded in Vestopal (M. Jaeger, Vésenaz, Geneva, Switzerland). Sections were stained with uranyl acetate and lead citrate and examined with a JEOL 200 CX electron microscope (M. Jaeger).

Assay of Calcitonin and Thyroglobulin in Mediastinal Metastatic Tumor Tissue

Calcitonin was measured with a double antibody radioimmunoassay according to Thorell *et al.*,¹⁷ with an antiserum against human synthetic calcitonin raised in goat (MILAB, Box 20047, Malmö, Sweden). Thyroglobulin was measured with a double antibody radioimmunoassay according to van Herle *et al.*¹⁸ Purified human thyroglobulin (a gift from Dr. van Herle, Division of Endocrinology, Department of Medicine, UCLA, Los Angeles, CA) was used to raise the antiserum (MILAB, Malmö, Sweden).

The metastatic tumor tissue (3 g) was homogenized for 2 minutes with a polytron PTA 10-35 (Kinematic, Luzern, Switzerland) in a protease inhibitor "cocktail" contained in 2 ml/g tissue of a 0.0035 M phosphate

buffer, pH 7.0, with 0.15 M NaCl and 0.02% NaN_3 ; Leupeptide (Peninsula, USA) 50 $\mu\text{g}/\text{ml}$, pepstatin (Sigma Chemical Co., St. Louis, MO) 50 $\mu\text{g}/\text{ml}$, PMSF (phenylmethylsulphonyl fluoride, Sigma Chemical Co.) 0.5 M in methanol (1 mM solution), benzamidine (Sigma Chemical Co.) 1 mM, TPCK (1-tosyl-amid-2-phenylchloromethylketone, Sigma Chemical Co.) 50 $\mu\text{g}/\text{ml}$, soybean-inhibitor (Sigma Chemical Co.) 50 $\mu\text{g}/\text{ml}$, DFP (di-iso-propyl-fluorophosphate; Fluca, FGR) 1 mM and trasylol (Bayer, FGR).

The suspension was stirred, using a magnetic stirrer, for 2 hours at 4°C, subsequently filtered through gauze and centrifuged for 10 min at +4°C and 10,000 $\times$ g. Calcitonin and thyroglobulin were analyzed in the tumor extract.

Results

Light Microscopy

Histologically, the thyroid tumor was diffusely outlined. It had infiltrated into the surrounding parenchyma, growing between the spared thyroid follicles and invading lymph and blood vessels. The tumor was very cellular with lobulated aggregates of closely packed epithelial cells, partly surrounded by thin connective tissue septa (Fig. 1).

The tumor cells were often oriented around lumina, thus forming cribriform structures which might resemble deranged follicles (Fig. 2). Some of these lumina contained eosinophil colloid-like fluid, often with marginal vacuoles close to the lining tumor cells, a picture which was reminiscent of that seen in thyrotoxicosis (Fig. 3). In other areas, the cells had formed compact clusters or anastomosing trabeculae, some of which contained palisading cylindrical tumor cells, arranged at right angles to the stromal septa (Fig. 4). The cells lining the follicle-like structures had the same appearance as those forming the compact clusters. Occasional true follicles, surrounded by basal laminae and stroma and lined by benign-looking epithelium, were also seen within the tumor. The tumor cells were polymorphic with marked nuclear atypia such as varying size, coarse chromatin structure and plump nucleoli. The cytoplasm was weakly eosinophil and granular. Some cells were vacuolated and had rather distinct cell boundaries. Many mitoses were seen and necrosis was frequently found. Occasional small eosinophil clumps were observed, intermingled with the tumor cells, but no material was found which stained with alkaline Congo red. More than one half of the tumor cell population showed varying amount of intracytoplasmic argyrophil granules (Fig. 5) of the type seen in endocrine cells producing polypeptide hormones and/or monoamines. The argyrophil cells were rather evenly distributed within the tumor and were found both

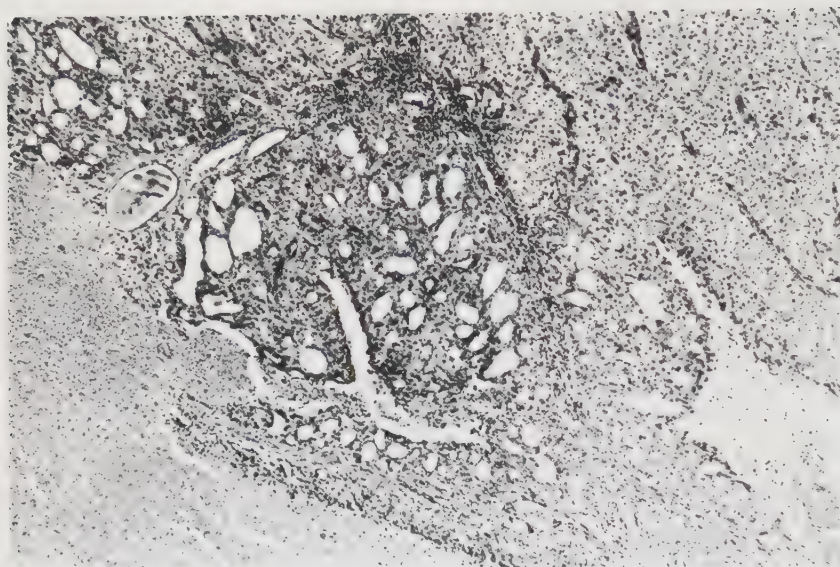


FIG. 1. The thyroid tumor showing cribriform structures and lobulated aggregates of closely packed tumor cells and separated by thin stromal septa. Necrotic area to the lower left (H & E, $\times 100$).

in the solid cell aggregates and among cells, lining the follicle-like structures. The tumor cells were nonargyrophilic and were devoid of 5-hydroxytryptamine. The histologic structure, as well as the results with the specialized techniques used, were the same in the primary tumor as in the metastases studied.

Immunohistochemistry

Sections from the primary tumor, as well as from the cervical metastases, showed numerous tumor cells with

material reacting with antibodies against somatostatin (Fig. 6A). An about equally large number of cells harbored neurotensin immunoreactive material. Both neurotensin antisera gave similar results. The number and distribution of the somatostatin and neurotensin containing cells appeared to be similar to that of the argyrophil cells. No immunoreactive calcitonin was found in the tumor tissue.

In addition, a large number of the tumor cells contained material which reacted with antibodies against

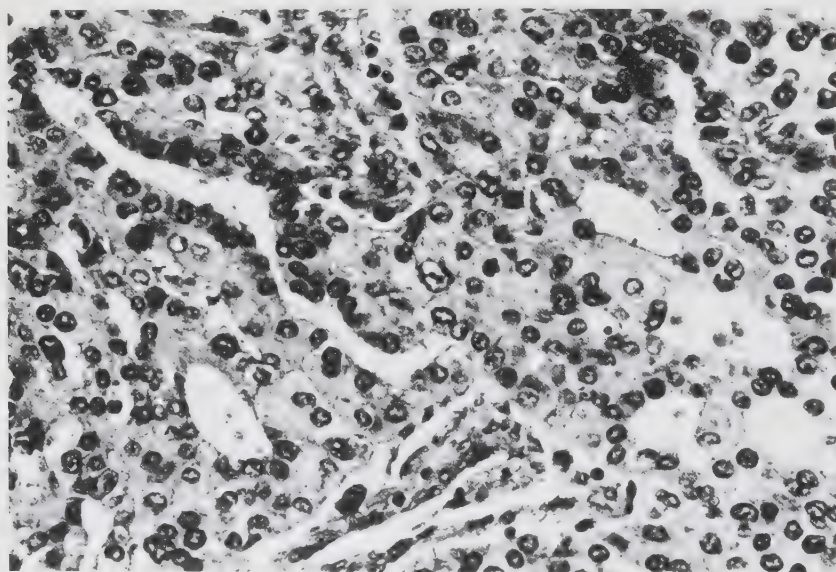
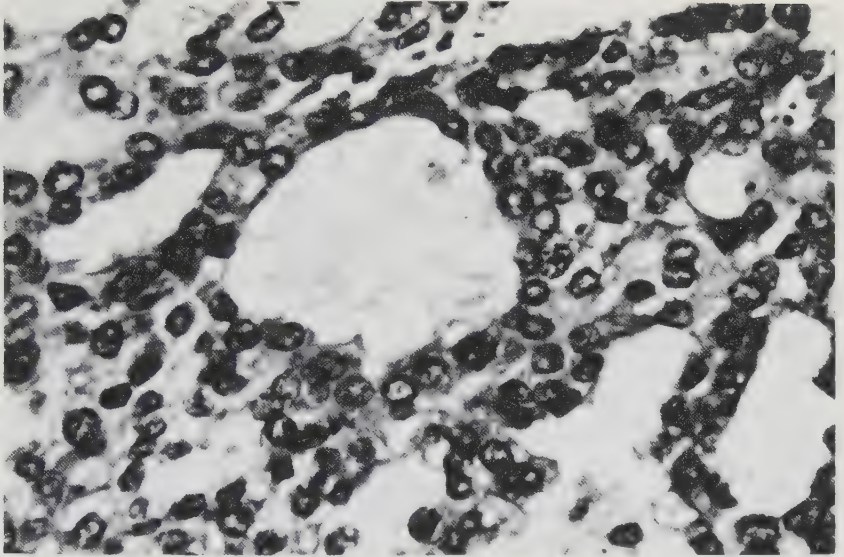


FIG. 2. Cribriform pattern. Tumor cells oriented around slit-like or round lumina which may resemble deranged thyroid follicles (H & E, $\times 450$).

FIG. 3. Follicle-like structure formed by tumor cells and containing colloid-like fluid with marginal vacuoles. There are no stromal septa separating the "follicles" (H & E, $\times 640$).



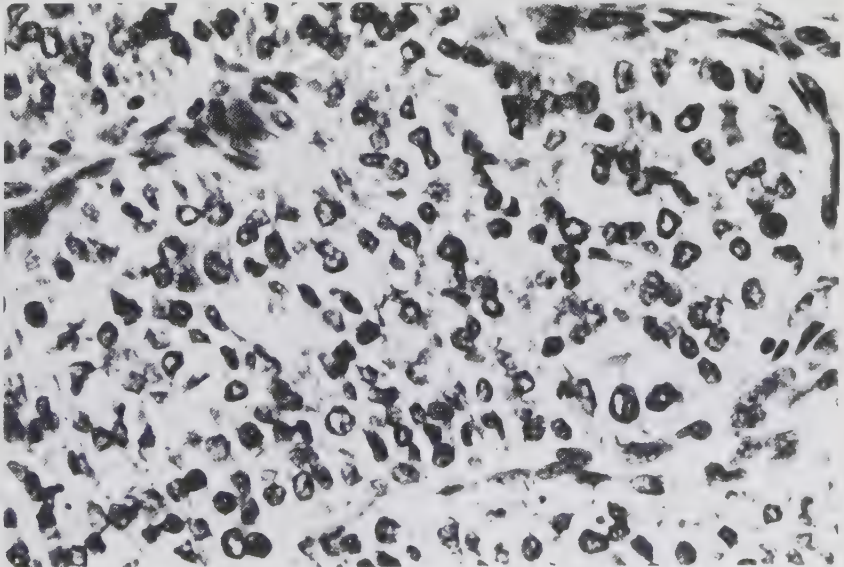
thyroglobulin (Fig. 7). These cells were rather unevenly distributed but were present within solid as well as cribriform areas. Thyroglobulin immunoreactive material was also observed within lumina of the cribriform structures.

Comparable results were obtained with the IF and PAP methods. Sections from tissues used as positive controls all showed specific immunostaining with their relevant antisera. No specific staining was observed in

sections incubated with antisera inactivated by their homologous antigens or in sections incubated with non-immune rabbit serum instead of the specific antiserum.

Cross-absorption studies: The absorption of the thyroglobulin antiserum with calcitonin, somatostatin or neurotensin did not result in any appreciable diminution of staining. No immunoreactivity for calcitonin, somatostatin or neurotensin was detected in the control series of differentiated follicular and papillary thyroid

FIG. 4. Compact clusters of tumor cells with palisading cylindrical cells marginally, arranged at right angles to the surrounding stromal septa (H & E, $\times 512$).



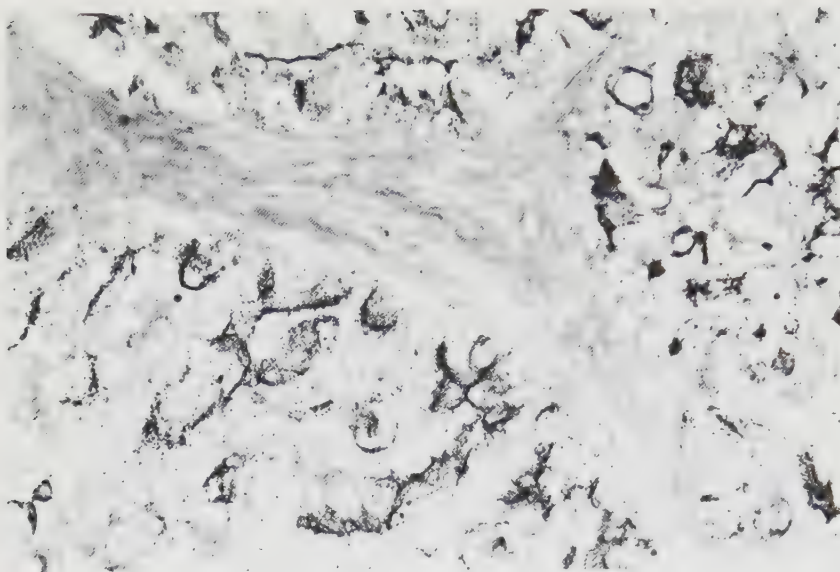


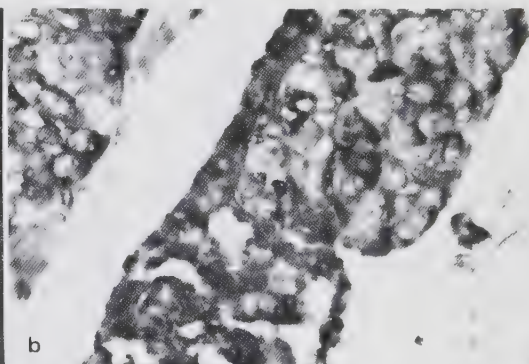
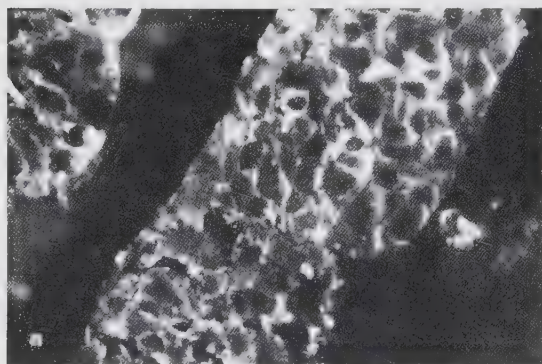
FIG. 5. Numerous argyrophil tumor cells. The intracytoplasmic argyrophil granules tend to be concentrated in the peripheral parts of the cytoplasm. Grimeilus' silver nitrate procedure ($\times 512$).

carcinoma, neither was any thyroglobulin immunoreactivity found in the tumor cells of the medullary thyroid carcinomas investigated.

Double immunostaining: A large number of tumor cells showing thyroglobulin immunoreactivity were also stained with antisera to somatostatin or neurotensin (Figs. 6A and 6B). It was not possible to determine whether immunoreactivity to all three antigens occurred in one and the same tumor cell. A small population of tumor cells showed specific staining for only one of the three hormones. Some tumor cells did not stain with any of the antisera used.

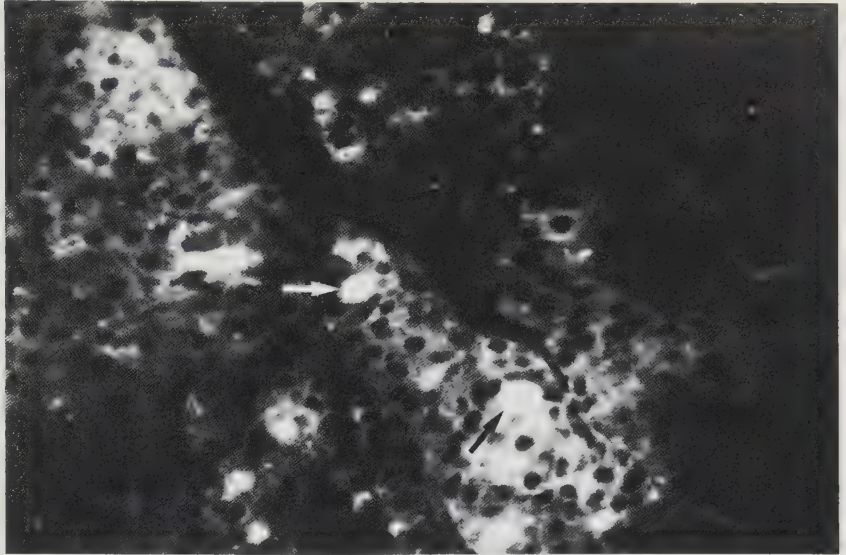
Electron Microscopy

The ultrastructure of the tumor cells was rather poorly preserved. Sections from blocks of the primary tumor and a cervical metastasis contained what appeared to be two structurally different types of tumor cell (Figs. 8A and 8B). One type showed rather numerous electron dense round bodies, ranging from 90 to 200 nm, some of which were surrounded by a thin membrane (Fig. 8A). Other organelles were mostly disintegrated, although a rich rough endoplasmic reticulum could be identified, as well as scattered, preserved mitochondria.



FIGS. 6A AND 6B. (A) Cluster of tumor cells within lumen of a blood vessel. A large number of tumor cells contain immunoreactive somatostatin. Indirect immunofluorescence using antibodies to synthetic human somatostatin (original magnification $\times 800$). (B) The same section as in Fig. 6A, processed according to the PAP method with antibodies to human thyroglobulin after preceding removal of the antibodies to somatostatin. Many tumor cells show specific immunoreaction to both thyroglobulin and somatostatin (compare with Fig. 6A). Diaminobenzidine substrate. Nuclei stained with hematoxylin (original magnification $\times 800$).

FIG. 7. Thyroglobulin immunoreactivity in a large proportion of the tumor cell population. Material within lumina of the cribriform structures also shows a strong specific fluorescence (arrows). Cervical lymph node metastasis. Indirect immunofluorescence ($\times 690$).



The other type of tumor cell was devoid of granules of the kind described above. These cells contained numerous vacuoles and cisternae containing a floccular or granular material (Fig. 8B). The profiles were mostly covered by small electron dense granules, resembling ribosomes and appeared to represent profiles of a rich vesicular rough endoplasmic reticulum. Cells belonging to this type also showed scattered varied-sized dense bodies with an appearance consistent with lysosomes.

Biochemical Findings

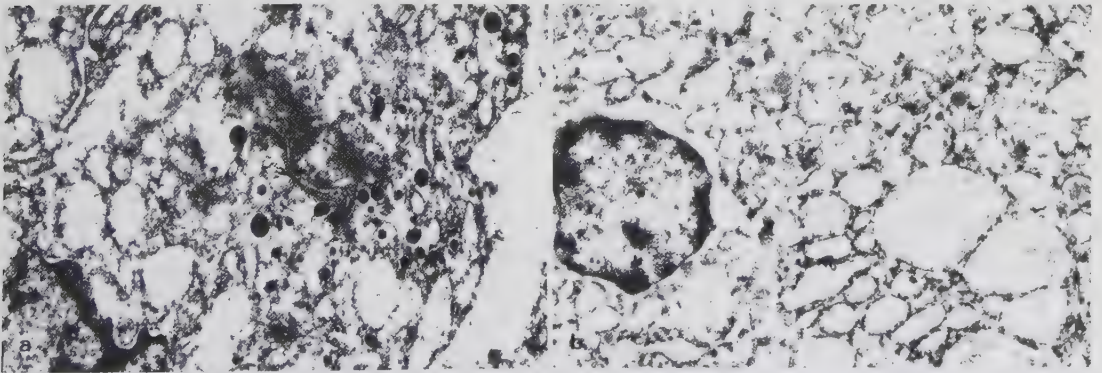
Extract of the metastatic tumor contained 66,000 μg thyroglobulin per litre corresponding to 76 μg thyro-

globulin per gram of tumor tissue. Immunoreactive calcitonin was not found in the tumor extract.

Discussion

The histologic, histochemical and immunologic investigations in this case revealed a previously unknown form of thyroid carcinoma, displaying features of both a follicular cell and parafollicular cell tumor.

Several features of the tumor may suggest a follicular nature. Thus the cribriform structures with intraluminal colloid-like material may indicate an attempt to reduplicate follicles. The demonstration of large amount of immunoreactive thyroglobulin in the patient's serum,



FIGS. 8A AND 8B. Electron micrographs of the two types of tumor cell in a cervical lymph node metastasis. (A) Part of a tumor cell with intracytoplasmic electron dense granules resembling secretory granules of endocrine type (original magnification $\times 20,800$). (B) Part of a tumor cell, showing a rich vesicular rough endoplasmic reticulum but no granules of endocrine type (original magnification $\times 22,600$).

the radioimmunologic and histochemical demonstration of immunoreactive thyroglobulin in the primary thyroid tumor and in its metastases, as well as the demonstration of an ^{131}I -uptake in at least some of the pulmonary metastases, are all features of differentiated thyroid carcinomas of follicular cell origin.¹⁹⁻²¹

On the other hand, the tumor also showed features which were reminiscent of a medullary carcinoma. The solid areas of the tumor, showing mosaic-like pattern and palisading of tumor cells, are suggestive of medullary carcinoma. Likewise, argyrophil cells are characteristic of this tumor. The argyrophil reaction has been widely used as a screening method for C-cells and their tumors. Argyrophil cells have not been described to occur in classic follicular or papillary thyroid carcinoma, nor have we been able to demonstrate such cells in a series of 30 cases of thyroid carcinoma which were strictly follicular or papillary in type or of the oxyphil variety. The argyrophil reaction reflects the presence of cytoplasmic secretory granules of the type seen in polypeptide hormone and/or monoamine secreting endocrine cells. Surprisingly however, no calcitonin immunoreactivity was found by the histochemical methods used, nor was this hormone found in extracts from metastatic tumor tissue. Furthermore, the patient's serum calcitonin levels were found to be within normal limits. Also, the tumor was devoid of amyloid deposits. On the other hand, a large proportion of the tumor cells contained immunoreactive somatostatin and many tumor cells also contained material reacting with antibodies against neurotensin. The presence of such material could explain the argyrophil reaction found in the cells. It is well established that human medullary carcinoma, besides secreting calcitonin, is also capable of producing various other polypeptides, including somatostatin. Somatostatin and neurotensin have also been reported in rat medullary thyroid carcinoma,^{22,23} although neurotensin has not, so far, been found in its human counterpart.²⁴ Somatostatin has likewise been found in normal parafollicular cells of certain mammals, although it has not been clearly established whether this hormonal peptide occurs in the same type of cell as calcitonin, *i.e.* C-cells, or in a distinct subpopulation of the polypeptide hormone producing parafollicular cell system of the thyroid gland.^{25,26} Whether or not a thyroid neoplasm producing somatostatin and/or neurotensin but no calcitonin should be classified as a medullary carcinoma then, is debatable and a matter of definition. However, it appears justified to designate such a neoplasm as a parafollicular cell tumor.

The immunoreactivity of this tumor shows distinct differences compared to the thyroglobulin-like immunoreactivity of medullary thyroid carcinomas reported by Kameda *et al.*²⁷ They have described an immunoreactivity of medullary carcinomas against a large molecular form of thyroid proteins. Their antiserum also

reacted weakly with follicular cells, and it contained one antibody population which could be neutralized with the major thyroglobulin fraction. However, that antibody population was distinctly different from that reacting with the C-cells, and no evidence was produced that linked the immunoreactivity of the C-cells to thyroglobulin. The immunoreactivity of the C-cells described by Kameda *et al.* was not directed against the major thyroglobulin fraction. The tumor described here showed distinct immunoreactivity with antibodies to the major thyroglobulin fraction and no reaction with the calcitonin antibodies. In addition, the antiserum to thyroglobulin used in this study did not cross-react with synthetic human calcitonin, somatostatin or neurotensin, nor did our calcitonin, somatostatin or neurotensin antisera cross-react with thyroglobulin. These findings, together with the demonstration of extremely high serum thyroglobulin levels and of ^{131}I -uptake in metastases of the tumor suggest that the material found to react with the antiserum to thyroglobulin was in fact thyroglobulin, biologically unrelated to polypeptide hormone synthesis.

Our results indicate that the tumor in this case represents a structurally and functionally biphasic tumor, containing elements of both follicular and parafollicular cells. The ultrastructural features of the tumor, showing two types of tumor cell, support this view. In this respect, the tumor may resemble a case reported by Bussolati and Monga,²⁸ showing light microscopic, histochemical and biochemical features consistent with a calcitonin-producing, amyloid-rich medullary carcinoma but with atypical microfollicular structures and electron microscopic patterns which were reminiscent of a follicular carcinoma. In their case however, the presence of thyroglobulin in the tumor or in the circulation was not investigated.

With regard to the histogenesis of the tumor, it may be debatable whether the tumor cells are derived from two functionally and embryogenetically distinct cell types, *i.e.*, the follicular cells and the parafollicular cells (or a subpopulation thereof), or from a common stem cell being the progenitor of both of these cell types. The first mentioned possibility would presuppose a coincidental malignant change in both cellular types. It may be mentioned that in the rat, radiation may cause neoplastic changes in both follicular cells and parafollicular cells.²⁹ However, this has not been shown to occur in humans.³⁰ In this particular case, there was no evidence for any radiation treatment given to the neck region prior to the development of the thyroid neoplasm.

The alternative possibility, *i.e.*, a tumor derived from a single type of precursor cell, is supported by the demonstration of both thyroglobulin and neuropeptides in the same tumor cells. The problem then would be whether it should be regarded as: (1) a follicular cell carcinoma where some carcinoma cells also have the

ability to form neuropeptides; (2) a parafollicular cell carcinoma where some tumor cells also are capable of thyroglobulin production; or (3) a compound follicular-parafollicular cell carcinoma where some tumor cells have differentiated into follicular as well as parafollicular tumor cells and some have assumed characteristics of intermediate forms. The first two suggestions appear unlikely, since no evidence was found for the production of neuropeptides in the control series of strictly follicular and papillary thyroid carcinoma or of the production of thyroglobulin in the series of classic medullary thyroid carcinoma also studied. The third suggestion would presuppose that at least some follicular and parafollicular cells may have a common cell of origin. This would be in conflict with the current belief that follicular cells and parafollicular cells have distinct embryogenesis, being derived from endoderm and neuroectoderm respectively. Interestingly, the same principal histogenetic problem also applies to a compound neoplasm of the gut, called adenocarcinoid, and reported to occur in the appendix and the colon.^{31,32} This tumor shows carcinoid structures as well as glandular formations with mucin-secreting cells and both exocrine and endocrine structural characteristics have been demonstrated in the individual tumor cells. The exact embryogenesis of carcinoid tumors in the gut is uncertain, but it has recently been suggested that carcinoid cells in the gut arise from endoderm.³² This makes it easier to explain the occurrence of two cell types in the gut adenocarcinoid tumors. If endoderm is capable of developing neuroendocrine cells for the gut, it is conceivable that, in the case of the thyroid gland, endodermally derived stem cells, besides forming follicular cells, may also be able to develop neuroendocrine parafollicular cells.

ADDENDUM

Since this manuscript was accepted for publication, a similar case of thyroid carcinoma has been reported by Hales et al. (Cancer 1982; 50:1352-1359), showing medullary pattern admixed with areas of follicular differentiation as well as evidence for both calcitonin and thyroglobulin production.

REFERENCES

- Williams ED. Medullary carcinoma of the thyroid. In: De Groot LJ, Cahill FG Jr, Odell WD et al., eds. Endocrinology, vol 2. New York: Grune & Stratton, 1979; 777-792.
- Williams ED, Morales AM, Horn R. Thyroid carcinoma and Cushing's syndrome. *J Clin Pathol* 1968; 21: 129-135.
- Sundler F, Alumets J, Håkanson R, Björklund L, Ljungberg O. Somatostatin-immunoreactive cells in medullary carcinoma of the thyroid. *Am J Pathol* 1977; 88:381-386.
- Capella C, Bordi C, Monga G et al. Multiple endocrine cell types in thyroid medullary carcinoma. *Virchows Arch [Pathol Anat]* 1978; 377:111-128.
- Skrabanek P, Cannon D, Dempsey J, Kirrane J, Neligan M, Powell D. Substance P in medullary carcinoma of the thyroid. *Experientia* 1979; 35:1259.
- Falck B, Ljungberg O, Rosengren E. On the occurrence of monoamines and related substances in familial medullary thyroid carcinoma with pheochromocytoma. *Acta Pathol Microbiol Scand* 1968; 74:1-10.
- Ljungberg O. On medullary carcinoma of the thyroid. *Acta Pathol Microbiol Scand(A)* 1972; 80 (Suppl 231):1-57.
- Voelkel EF, Tashjian AH Jr, Davidoff FF, Cohen RB, Perlia CP, Wurtman RJ. Concentrations of calcitonin and catecholamines in pheochromocytomas, a mucosal neuroma and medullary thyroid carcinoma. *J Clin Endocrinol* 1973; 37:297-307.
- Dymling J-F, Ljungberg O, Hillyard CJ, Greenberg P, Evans IMA, MacIntyre I. Whisky: A new provocative test for calcitonin secretion. *Acta Endocrinol (Copenh)* 1976; 82:500-509.
- Puchtler H, Sweat F, Levine M. On the binding of Congo red by amyloid. *J Histochem Cytochem* 1962; 10:355-364.
- Romeis B. *Mikroskopische Technik*, ed. 15. München: R Oldenbourg, 1948; 264.
- Pearse AGE. *Histochemistry*, ed. 2. London: J & A Churchill Ltd, 1960; 922.
- Grmelius L. A silver nitrate stain for α_2 -cells in human pancreatic islets. *Acta Societatis Medicorum Upsaliensis* 1968; 73:243-270.
- Goldman M. Fluorescent antibody methods. New York: Academic Press, 1968.
- Sternberger LA. *Immunocytochemistry*, ed. 2. New York: John Wiley, 1979; 1-354.
- Tramu G, Pillez A, Leonardelli J. An efficient method of antibody elution for the successive or simultaneous localization of two antigens by immunocytochemistry. *J Histochem Cytochem* 1978; 26:322-324.
- Thorell JI, Larson SM. Methodology and clinical applications. In: Radioimmunoassay and Related Techniques. St Louis: CM Mosby, 1978; 202-203.
- Van Herle AJ, Uller RP, Matthews NL, Brown J. Radioimmunoassay for measurement of thyroglobulin in human serum. *J Clin Invest* 1973; 52:1320-1327.
- Burt A, Goudie RB. Diagnosis of primary thyroid carcinoma by immunohistological demonstration of thyroglobulin. *Histopathol* 1979; 3:279-286.
- Pacini F, Pinchera A, Giani C, Grasso L, Baschieri L. Serum thyroglobulin concentrations and ¹³¹I whole body scans in the diagnosis of metastases from differentiated thyroid carcinoma (after thyroidectomy). *Clin Endocrinol* 1980; 13:107-110.
- Black EG, Gimlette TMD, Maisey MN, Cassoni A, Harmer CL, Oates CD. Serum thyroglobulin in thyroid cancer. *Lancet* 1981; II:443-445.
- Birnbaum RS, Muszynski M, Roos BA. Tumor and plasma somatostatin-like immunoreactivity in transplantable rat medullary thyroid carcinoma. *Cancer Res* 1980; 40:4192-4196.
- Zeytinoglu FN, Gagel RF, Tashjian AH Jr, Hammer RA, Lee-man SE. Characterization of neurotensin production by a line of rat medullary thyroid carcinoma cells. *Proc Natl Acad Sci USA* 1980; 77:3741-3745.
- Bača I, Schmidt-Gayk H. Neurotensin and medullary carcinoma of the thyroid gland. *J Cancer Res Clin Oncol* 1981; 100:229-230.
- Van Noorden S, Polak JM, Pearse AGE. Single cellular origin of somatostatin and calcitonin in the rat thyroid gland. *Histochemistry* 1977; 53:243-247.
- Kusumoto Y. Calcitonin and somatostatin are localized in different cells in the canine thyroid gland. *Biomedical Research* 1980; 1:237-241.
- Kameda Y, Harada T, Ito K, Ikeda A. Immunohistochemical study of the medullary thyroid carcinoma with reference to C-thyroglobulin reaction of tumor cells. *Cancer* 1979; 44:2071-2082.
- Bussolati G, Monga G. Medullary carcinoma of the thyroid with atypical patterns. *Cancer* 1979; 44:1769-1777.
- Triggs SM, Williams ED. Experimental carcinogenesis in the rat thyroid follicular and C-cells. *Acta Endocrinol (Copenh)* 1977; 85:84-92.
- Holm L-E. Incidence of malignant thyroid tumors in man after diagnostic and therapeutic doses of iodine-131: Epidemiologic and histopathologic study (Thesis). Stockholm, 1980; 1-157.
- Olsson B, Ljungberg O. Adenocarcinoid of the vermiform appendix. *Virchows Arch [Pathol Anat]* 1980; 386:201-210.
- Lyss AP, Thompson JJ, Glick JH. Adenocarcinoid tumor of the colon arising in preexisting ulcerative colitis. *Cancer* 1981; 48:833-839.

Effect of Therapy on the Serum Thyroglobulin Concentration in Patients with Toxic Diffuse Goitre, Toxic Nodular Goitre and Toxic Adenoma

by

U-B Ericsson, L Tegler, JF Dymling and JI Thorell

Departments of Internal Medicine, Endocrinology and Clinical Chemistry at Malmö General Hospital, University of Lund, Malmö, Sweden and the Department of Internal Medicine, University Hospital of Linköping, Sweden

SUMMARY

Serum thyroglobulin (S-Tg) was measured in 64 patients with thyrotoxicosis, 38 of whom had toxic diffuse goitre (Graves disease), in 19 with toxic nodular goitre and in 7 with toxic adenoma. Before treatment, most patients had increased S-Tg concentrations, regardless of what type of thyrotoxicosis they had. After therapy the course of the S-Tg varied, two major patterns being observed: the S-Tg concentration increased in some patients but decreased in others, although no relationship could be found between these patterns and the outcome of therapy. However, the median pre-treatment concentrations of S-Tg were significantly higher in patients with toxic nodular goitre and toxic adenoma than in those with toxic diffuse goitre ($p < 0.001$ and $p < 0.05$, respectively), but did not differ significantly between patients with toxic nodular goitre and toxic adenoma. The lowest post-treatment S-Tg concentrations were found after surgery, irrespective of type of thyrotoxicosis. The median pre-treatment and post-treatment S-Tg concentrations in patients with toxic diffuse goitre who relapsed, did not differ from those patients in remission. This was also true of patients with toxic nodular goitre. In both groups, however, there was a tendency towards higher pre-treatment S-Tg values in patients who subsequently relapsed. Serial determinations of S-Tg, on the other hand, are of limited value in predicting the risk of recurrence, independent of which type of thyrotoxicosis is involved

Thyroglobulin (Tg) is a normal secretory product of the thyroid gland. The mechanism for its release is still unclear as are its possible extrathyroidal physiological effects. Increased concentration of Tg in serum (S-Tg) is found in a variety of thyroid disorders (Hjort, 1963; Torrigiani et al., 1969; Pezzino et al., 1977; van

Herle et al., 1979; Endo et al., 1979; Eber et al., 1980; Benita et al., 1981; Roti et al., 1981), and most thyrotoxic patients have increased S-Tg concentrations (Torrighiani et al., 1969; van Herle et al., 1973; Ochi et al., 1975; Pezzino et al., 1977; Endo et al., 1979; Feldt-Rasmussen et al., 1979; Eber et al., 1980). It has been demonstrated in rats, that the intravenous injection of the long-acting, thyroid stimulator (LATS), induces the release of Tg into the circulation (van Herle et al., 1975); otherwise, the factors stimulating the release of Tg in toxic diffuse goitre (Graves' disease) and the other varieties of thyrotoxicosis are essentially unknown.

The pathophysiological significance of the elevated S-Tg concentration is unknown. The circulating Tg has not been shown to contribute to the thyrotoxic state.

Measurement of S-Tg has been advocated to evaluate the outcome of therapy in patients with thyrotoxicosis (Pezzino et al., 1977; Uller et van Herle, 1978; Gardner et al., 1979; Feldt-Rasmussen et al., 1982; Kawamura et al., 1983). It has been suggested that the S-Tg concentration present at the cessation of antithyroid drug treatment might serve as an indicator of future recurrence of the disease (Uller et van Herle, 1978; Gardner et al., 1979; Kawamura et al., 1983) though this hypothesis derives no support from the work of others (Feldt-Rasmussen et al., 1982).

The aim of this study was to prospectively evaluate the usefulness of the S-Tg measurements in predicting the risk of recurrence in patients with toxic diffuse goitre, toxic nodular goitre and toxic adenoma.

MATERIALS AND METHODS

Patients

Sixty-four patients with thyrotoxicosis were studied, diagnosis being based on clinical examination, serum T_3 - and T_4 concentrations, and in some cases on the results of a TRH stimulation test as well. A thyroid scintigram was performed in 57 of the patients. Thirty-eight

patients (5 men and 33 women, median 49 years, range: 17-74 years) had toxic diffuse goitre, 19 (2 men and 17 women, median 52 years, range: 32-76 years) were classified as toxic nodular goitre and 7 (1 man and 6 women, median 45 years, range: 33-62 years) as toxic adenomas. The patients in each group were further subdivided according to the therapy they received. Characteristics and treatment of the patients in the different groups are given in Table I.

Recurrence was defined as an exacerbation of the thyrotoxic state after a period of at least two months with clinical and laboratory euthyroidism. The category of patients not attaining euthyroidism after the first treatment was referred to as "insufficient primary treatment".

Serum samples for the determination of Tg were obtained before therapy, on several occasions during therapy (in the patients on long-term treatment with antithyroid drugs and thyroxine), between one and two months after therapy, and on several occasions during the follow-up period after therapy.

Thyroglobulin antibodies were measured by a passive haemagglutination test using the Wellcome kit (Thymune-T, Wellcome research laboratories, Beckenham, England). Only subjects with an antibody titre of less than 1:10 were included in the study. The Tg-antibody titre was controlled in each serum sample.

Thyroglobulin radioimmunoassay

S-Tg concentrations were measured with a double antibody radioimmunoassay using highly purified Tg, the details of which are reported elsewhere (Ericsson et al., 1984). Antiserum to human Tg was raised in rabbits and used at a final dilution of 1:250 000. Tg was enzymatically labelled with ^{125}I , using lactoperoxidase (Thorell and Johansson, 1971). The stock standard was stored at -20°C in normal goat serum. One hundred microlitres of the standard solution or the serum sample was preincubated for 48 hours at $+4^{\circ}\text{C}$ with 200 μl anti-serum. Two hundred microlitres ^{125}I (approx. 10 000 cpm) tracer solution was added and the tubes incubated for a further 72 hours at

+4°C, after which 50 µl goat anti-rabbit serum was added as precipitating antibody, together with 50 µl rabbit serum diluted 1:250. After being incubated once more, for 24 hours at +4°C, the tubes were centrifuged 15 min at +4°C and 2400 g, and the supernatants discarded. The precipitates were counted in a multi-crystal gamma counter (Nuclear Enterprises, NE 1600, Edinburgh, Scotland).

The minimum detectable concentration of S-Tg was 2 µg/l. The intra-assay coefficient of variation, estimated from duplicate determinations of samples with S-Tg concentrations between 10-200 µg/l, was 6.3%. The reproducibility was ascertained by inclusion of two different control sera in all assays. The results from the measurements of these sera over one year was 9.4 ± 1.5 µg/l and 29.5 ± 4.1 µg/l (mean $\pm$ SD).

Cross-reactivity was tested for T₃, T₄, MIT and DIT. Amounts of up to 1000 µg/l did not interfere with the binding of ¹²⁵I-Tg. The reference values for S-Tg were established in samples from 400 females (48 or 53 years old) without evidence of thyroid disease, recruited from a health screening program at the Department of Preventive Medicine, Malmö. Their S-Tg values ranged from undetectable to 77 µg/l (median 13 µg/l). The 97.5th percentile was equal to 40 µg/l. This value was taken as the upper limit for the reference range.

Statistical methods

The Mann-Whitney test was used for unpaired observations and the Wilcoxon's test for paired observations.

RESULTS

Increased concentrations of S-Tg were most frequent in patients with toxic adenoma (6/7 patients, 86%), followed by patients with toxic nodular goitre (14/19 patients, 74%), the lowest incidence being found in patients with toxic diffuse goitre (23/38 patients, 60.5%). The median pre-treatment S-Tg concentrations were significantly higher in patients with toxic nodular goitre (79 µg/l) and toxic adenoma (70 µg/l) than in patients with toxic diffuse goitre (49 µg/l, $p < 0.001$ and $p < 0.05$, respectively, Fig. 1), but did not dif-

fer between toxic nodular goitre and toxic adenoma. There was no significant correlation between the pre-treatment S-Tg concentrations and T_3 or T_4 concentrations in any of the groups (toxic diffuse goitre: T_3 ; $r = 0.005$, T_4 ; $r = 0.27$, toxic nodular goitre: T_3 ; $r = 0.41$, T_4 ; $r = 0.43$ and toxic adenoma: T_3 ; $r = 0.25$, T_4 ; $r = 0.47$).

Table I summarizes the outcome of different forms of treatment and the S-Tg values before and after therapy in each patient group. The individual S-Tg concentrations in the same groups before, during and after treatment, are given in Figures 2-4.

Ten out of thirty-eight patients (26%) with toxic diffuse goitre had a relapse between 2 and 34 months (median 9 months) after therapy. The median S-Tg concentrations did not differ between the remission and the exacerbation groups either before, during or after therapy. Individual S-Tg concentrations showed no distinct pattern, both increased, decreased, and essentially unchanged values being found among the patients who continued in remission as well as among those who had a relapse. S-Tg concentrations decreased after therapy in 4/5 patients who continued in remission. In the group treated with radioiodine, 2/4 patients who relapsed and 3/6 in remission showed decreased S-Tg concentrations. In all patients treated surgically S-Tg concentrations decreased postoperatively.

Table II summarizes the characteristics of those patients with toxic diffuse goitre who relapsed after treatment with antithyroid drugs and thyroxine or ra-

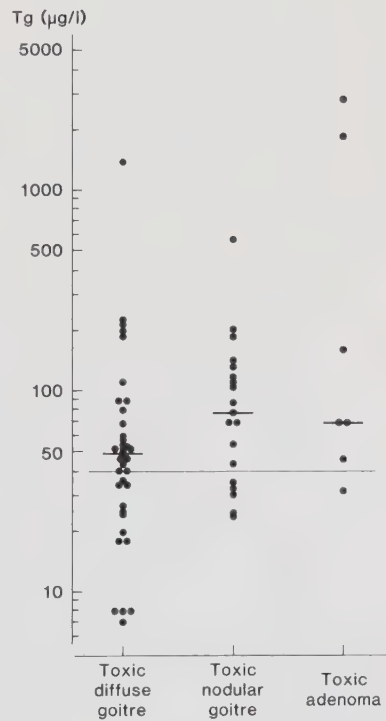


Fig. 1. Pre-treatment serum thyroglobulin concentrations in patients with toxic diffuse goitre, toxic nodular goitre and toxic adenoma. — denotes the median serum thyroglobulin concentration and — denotes the upper limit of the normal range.

Table I. Median S-Tg concentrations and outcome of therapy in the various categories of thyrotoxicosis in relation to type of treatment

	TOXIC DIFFUSE GOITRE			TOXIC NODULAR GOITRE			TOXIC ADENOMA	
	T r e a t m e n t			T r e a t m e n t			T r e a t m e n t	
	Surgery	Radio-iodine	Antithyroid drugs	Surgery	Radio-iodine	Antithyroid drugs	Surgery	Radio-iodine
Patients	No	9	17	12	8	7	4	6
Recurrence	No	1	4	5	0	1	2	0
Insufficient primary treatment	No	0	7	0	0	3	0	0
Hypothyroidism post treatment	No	2	12	0	0	5	0	0
S-Tg before treatment median + total range	µg/l	50 (24-1390)	52 (8-221)	44.5 (7-1060)	83.5 (24-187)	105 (31-203)	34.5 (25-574)	117 (32-2960)
S-Tg after treatment median + total range	µg/l	19 (3-44)	48 (25-151)	22 (2-112)	27 (5-60)	70 (32-136)	34 (27-44)	38.5 (8-81)
Observation time after primary treatment	months	50 (16-62)	29 (7-58)	14.5 (9-51)	48 (24-52)	23 (8-40)	24 (16-37)	25 (9-62)

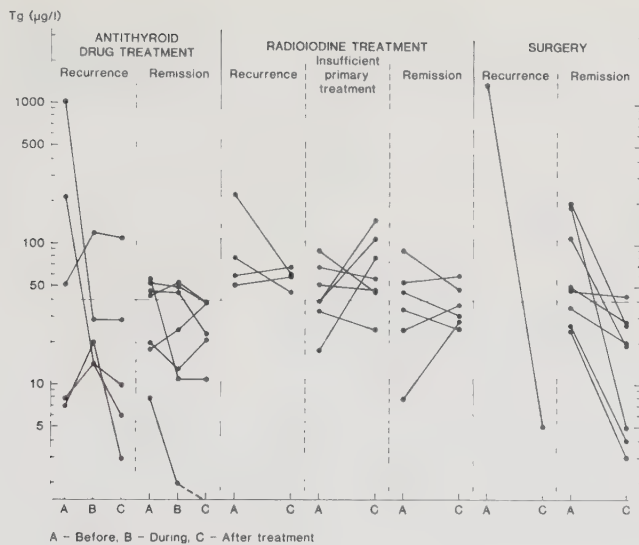


Fig. 2. The individual course of the serum thyroglobulin concentration in patients with toxic diffuse goitre treated with antithyroid drugs (in combination with thyroxine), radioactive iodine and surgery. — denotes the upper limit of the normal range.

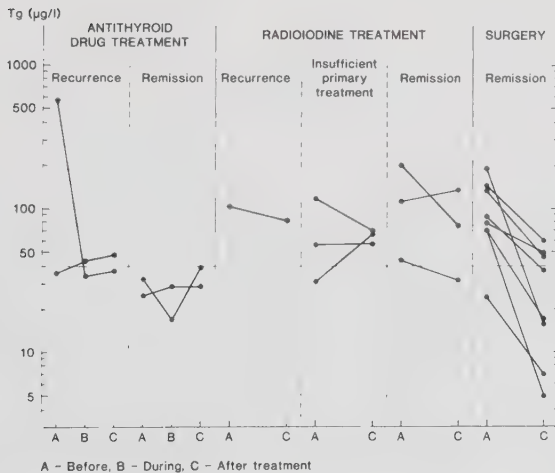


Fig. 3. The individual course of the serum thyroglobulin concentration in patients with toxic nodular goitre treated with antithyroid drugs (in combination with thyroxine), radioactive iodine and surgery. — denotes the upper limit of the normal range.

dioactive iodine in comparison with those who continued in remission. There was no significant difference between the median pre-treatment or post-treatment S-Tg concentrations in the two groups.

Of the nineteen patients with toxic nodular goitre, three (16%) had a relapse 2, 8 or 30 months after therapy. The same variations in individual S-Tg patterns were found as among the patients with toxic diffuse goitre, there being no difference in this respect between the patients in remission and those who subsequently relapsed. The S-Tg concentration decreased after therapy in 2/3 patients who relapsed and in 10/13 in remission.

None of the patients with toxic adenoma had a relapse during the obser-

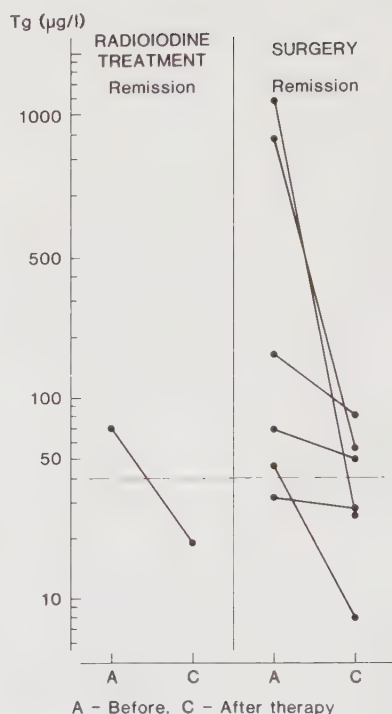


Fig. 4. The individual course of the serum thyroglobulin concentration in patients with toxic adenoma treated with radioactive iodine and surgery. — denotes the upper limit of the normal range.

Table II. Relation of serum thyroglobulin to relapse of thyrotoxicosis in patients with toxic diffuse goitre

		Antithyroid drug treatment		Radio-iodine treatment	
		Remission	Recurrence	Remission	Recurrence
Patients	No	7	5	6	4
Tg before treatment	µg/l	43	52	40	70.5
median + total range		(8-57)	(7-1060)	(8-91)	(52-221)
Tg after treatment	µg/l	23	10	34	61
median + total range		(2-38)	(3-112)	(26-60)	(47-69)
Observation period	months	14	19	13.5	26.5
after treatment		(9-51)	(12-36)	(7-50)	(12-56)
Duration of treatment	months	18	18	---	---
		(9-25)	(6.5-24)		

vation period after therapy. The S-Tg concentration decreased after treatment in all 7 patients.

DISCUSSION

The observation previously reported that most hyperthyroid patients have increased S-Tg concentrations (Torrighiani et al., 1969; van Herle et al., 1973; Ochi et al., 1975; Pezzino et al., 1977; Endo et al., 1979; Feldt-Rasmussen et al., 1979; Eber et al., 1980; Roti et al., 1981) is confirmed by our findings. Unlike Uller et van Herle (1978), however, we found a marked overlap in S-Tg concentration between normal and hyperthyroid subjects, also been reported by others (Ochi et al., 1975; Endo et al., 1979; Feldt-Rasmussen et al., 1979; Eber et al., 1980). van Herle and coworkers suggested that the increased S-Tg concentrations seen in toxic diffuse goitre may be the result of Tg being released from the thyroid gland when it is stimulated by the LATS (van Herle et al., 1975). On the other hand, increased S-Tg concentrations were more frequent in toxic adenoma, where no stimulation by LATS occurs, rather than in toxic diffuse goitre. Hjort et al. (1974), however, found no difference between the S-Tg concentrations in patients with and without LATS activity.

The reported effects of antithyroid drug treatment on the serum concentrations of S-Tg in toxic diffuse goitre have varied. Pezzino et al. (1977) reported reduced S-Tg in 10/12 patients following antithyroid drug treatment. In both the exacerbation and the remission groups of their 19 patients Uller et van Herle (1978) found both increased, decreased, and essentially unchanged S-Tg concentrations at the end of therapy as compared with their pre-treatment levels. They did, however, find higher S-Tg concentrations at the end of therapy in the exacerbation group than in the remission group, though pre-treatment S-Tg concentrations had already been high in the exacerbation group, and changed very little during the course of the antithyroid drug treatment.

Like Uller et van Herle (1978) we found a variable course of the S-Tg concentration among our patients with toxic diffuse goitre, irrespective of whether they continued in remission or relapsed, but there

was no correlation between the course of S-Tg and the outcome of therapy. Neither was there any significant difference between the pre-treatment and post-treatment S-Tg concentrations, either in the 7 patients in remission or the 5 who relapsed. There was, however, a tendency towards higher pre-treatment S-Tg concentrations in the exacerbation group.

On the basis of their results, Uller et van Herle suggested that the serum concentration of Tg at the end of therapy might be a useful clinical index of the outcome in patients treated with antithyroid drugs. Their results have been confirmed by Gardner et al. (1979), and more recently by Kawamura et al. (1983), but could not be reproduced by Feldt-Rasmussen et al. (1982).

As with the patients with toxic diffuse goitre on antithyroid drug treatment, in those treated with radioactive iodine we found no significant difference in the median pre-treatment or post-treatment S-Tg values between the exacerbation group and the remission group, which is in agreement with the findings of Pezzino et al. (1977).

The lowest post-treatment S-Tg concentrations were found among the patients treated with surgery, and most likely caused by the reduction of the number of thyroid follicular cells. In addition, the frequency of recurrences was lowest among the patients treated with surgery. It is unclear why the patients treated with radioactive iodine, who also reduced their functioning thyroid cell mass, do not show a similar decrease in their post-treatment S-Tg concentrations. Hypothetically, however, the radioactive iodine may permanently damage the "secretion" mechanism.

Since the varying S-Tg pattern observed in all groups of thyrotoxicosis showed no relation to the T_3 or T_4 concentrations present in individual patients, the variation in the S-Tg level is not directly related to the degree of severity of the disease.

The first treatment dose of radioactive iodine was often insufficient to induce euthyroidism in the hyperthyroid patients. After the first

dose of radioactive iodine the S-Tg concentrations changed very little or rose, compared with the pre-treatment value in those patients who required further doses.

In conclusion, the course of the S-Tg concentration is variable in patients with thyrotoxicosis and has no predictive value apart from a slight tendency towards higher pre-treatment S-Tg concentrations in the patients who subsequently relapsed. The post-treatment S-Tg concentration is of limited value in predicting the risk of recurrence.

REFERENCES

1. Benita, G., Lapalus, F., Bayer, V., Bornet, H. & Hoch, M. (1981) Radioimmunoassay of serum thyroglobulin. Technique and clinical results. *Eur J Nucl Med*, 6, 515-520.
2. Eber, M., Abecassis, J., Grob, J.C., Ott, G. & Methlin, G. (1980) Immunoradiometric assay for human thyroglobulin and variations in thyroid pathology. *Clin Chim Acta*, 105, 51-58.
3. Endo, Y., Nakano, J., Ohtaki, S., Izumi, M., Hamaguchi, Y., Yoshitake, S. & Ishikawa, E. (1979) An enzyme immunoassay for the measurement of thyroglobulin in human serum. *Clin Chim Acta*, 95, 325-336.
4. Ericsson, U-B., Larsson, I. & Thorell, J.I. (1984) Purification and storage of thyroglobulin. Two important factors influencing the radioimmunoassay for thyroglobulin. *Scand J Clin Lab Invest*, In press.
5. Feldt-Rasmussen, U., Bech, K. & Date, J. (1979) Serum thyroglobulin in patients with toxic and non-toxic goitres compared to sex- and age-matched control subjects. *Acta Endocrinol (Kbh)*, 91, 264-270.
6. Feldt-Rasmussen, U., Bech, K., Date, J., Hyltoft Petersen, P. & Johansen, K. (1982) A prospective study of the differential changes in serum thyroglobulin and its autoantibodies during propylthiouracil or radioiodine therapy of patients with Graves' disease. *Acta Endocrinol (Kbh)*, 99, 379-385.

7. Gardner, D.F., Rothman, J. & Utiger, R.D. (1979) Serum thyroglobulin in normal subjects and patients with hyperthyroidism due to Graves' disease: Effects of T_3 , iodide, ^{131}I and antithyroid drugs. *Clin Endocrinol (Oxf)*, 11, 585-594.
8. Hjort, T. (1963) "Thyroglobulin" in the serum. *Acta Med Scand*, 174, 137-146.
9. Hjort, T., Friis, Th., Birk Lauridsen, U. & Persson, I. (1974) Thyroglobulin in serum after TSH stimulation in hyperthyroidism. *Acta Endocrinol (Kbh)*, 75, 699-706.
10. Kawamura, S., Kishino, B., Tajima, K., Mashita, K. & Tarui, S. (1983) Serum thyroglobulin changes in patients with Graves' disease treated with long term antithyroid drug therapy. *J Clin Endocrinol Metab*, 56, 507-512.
11. Ochi, Y., Hachiya, T., Yoshimura, M., Miyazaki, T., Majima, T., Kaimatsu, I. & Takahashi, H. (1975) Radioimmunoassay for estimation of thyroglobulin in human serum. *Endocrinol Japon*, 22, 351-356.
12. Pezzino, V., Cozzani, P., Filetti, S., Galbiati, A., Lisi, E., Squatrito, S. & Vigneri, R. (1977) A radioimmunoassay for human thyroglobulin: methodology and clinical applications. *Eur J Clin Invest* 7, 503-508.
13. Roti, E., Robuschi, G., Emanuele, R., Bandini, P. & Gnudi, A. (1981) Radioimmunoassay of thyroglobulin in human serum: Concentrations in normal subjects and in patients with thyroid disease. *J Nucl Med Allied Sci*, 25, 57-63.
14. Thorell, J.I. & Johansson, B.G. (1971) Enzymatic iodination of polypeptides with ^{125}I to high specific activity. *Biochim Biophys Acta*, 251, 363-369.
15. Torrigiani, G., Doniach, D. & Roitt, I.M. (1969) Serum thyroglobulin levels in healthy subjects and in patients with thyroid disease. *J Clin Endocrinol Metab*, 29, 305-314.
16. Uller, R.P. & van Herle, A.J. (1978) Effect of therapy on serum thyroglobulin levels in patients with Graves' disease. *J Clin Endocrinol Metab*, 46, 747-755.
17. van Herle, A.J., Uller, R.P., Matthews, N.L. & Brown, J. (1973) Radioimmunoassay for measurement of thyroglobulin in human serum. *J Clin Invest*, 52, 1320-1327.

18. van Herle, A.J., Klandorf, H. & Uller, R.P. (1975) A radioimmunoassay for serum rat thyroglobulin. Physiologic and pharmacological studies. J Clin Invest, 56, 1073-1081.
19. van Herle, A.J., Vassart, G. & Dumont, J.E. (1979) Control of thyroglobulin synthesis and secretion. N Engl J Med, 301, 307-314.

The Prevalence of Thyroglobulin Antibodies in Adults with and without Thyroid Disease as Measured with a Sensitive Solid-Phase Immunosorbent Radioassay

by

Ulla-Britt Ericsson, Svend Borup Christensen and Jan I Thorell

Departments of Internal Medicine, Surgery and Clinical Chemistry,
Malmö General Hospital, University of Lund, Malmö Sweden

ABSTRACT

Sera from 228 patients with thyroid disease and 140 healthy subjects without clinical or biochemical evidence of thyroid disease, were tested using a sensitive solid-phase immunosorbent radioassay (RIA) and a passive haemagglutination test (TRC test) for thyroglobulin antibodies (Tg-ab). With the RIA technique, Tg-ab was found in as many as 27% of the controls (36% of the women and 15% of the men), whereas only 0.7% of them were Tg-ab positive with the TRC test. All individuals with primary hyperthyroidism were Tg-ab positive with the RIA, compared with only 56% with the TRC test. Tg-ab (RIA) was found in 43/53 (81%) of the patients with toxic diffuse goitre, and in 30-40% of the patients with toxic nodular goitre, toxic adenoma, atoxic goitre and thyroid carcinoma, the TRC test being positive in 10-17% of these patients. The high prevalence of Tg-ab in the healthy population suggests that subclinical thyroiditis is more frequent than has been assumed from antibody measurements made with less sensitive methods, and is in agreement with the prevalences reported from autopsy studies.

INTRODUCTION

Although the presence of anti-thyroglobulin antibodies in the serum of patients with goitre, with and without associated hypo- or hyperthyroidism, has been considered evidence for the occurrence of chronic thyroiditis (Doniach et al., 1979), the clinical significance of anti-thyroglobulin and anti-microsomal antibodies in apparently healthy individuals is the subject of controversy. Post-mortem studies have shown lymphocytic infiltration in the thyroid gland in patients who had positive serum anti-thyroglobulin or complement-

Key words: Thyroglobulin autoantibodies. Immunosorbent radioassay. Thyroiditis

fixing antithyroid microsomal antibodies but not overt thyroid disease (Goudie et al., 1959; Bastenie et al., 1967). On the other hand, some cases with morphologically diagnosed thyroiditis escaped serological detection using the haemagglutination or complement-fixing methods (Goudie et al., 1959; Loeb et al., 1973).

Generally, thyroglobulin antibodies (Tg-ab) have been found in 2 to 5% of adults without known thyroid disease, using the haemagglutination technique (Hill et al., 1961; Whittingham et al., 1969; Borup Christensen et al., 1984), although there are a few reports of higher prevalences (Hackett et al., 1960; Dingle et al., 1966). With the more sensitive radioimmunological (RIA) methods, the frequencies reported in healthy individuals have varied from 4 to 15% (Salabè et al., 1972; Peake et al., 1974; Date et al., 1980). The wide variations found in the frequency of Tg-ab positive sera may be due to variations in the sensitivity of the assays, as well as in the selection of the individuals composing the groups studied.

The aim of the present investigation was to compare the prevalence of Tg-ab as measured with a new sensitive solid-phase immunosorbent radioassay with that found in a conventional passive haemagglutination test (TRC test). Patients with different thyroid disorders were included and compared with a control group which clinical investigation and extensive laboratory analysis had shown to be free from thyroid disease.

MATERIAL AND METHODS

Study subjects

The study is based on the observation of a total of 368 individuals (271 women and 97 men), who were subdivided into 8 different groups according to the clinical diagnosis. The control groups (80 women and 60 men) were recruited from a large health screening programme at the Department of Preventive Medicine, Malmö. The women were part of a population study on the prevalence of thyroid disorders (Borup Christensen et al., 1984). All individuals had normal thyroid glands on palpation. They had no history of thyroid disease and showed normal serum T_3 and T_4 concentrations as well as normal serum TSH and thyro-

globulin concentrations. In the patient groups, the diagnosis was based on clinical findings, thyroid scan and the results of measurements of T_3 , T_4 and TSH concentrations and in some cases on the results of a TRH stimulation test. A histopathologic or cytologic diagnosis was obtained in all patients with atoxic goitre as well as in all patients with thyroid carcinoma. Among the hypothyroid patients morphological diagnosis was obtained in 10/27 cases, chronic thyroiditis being found in nine of them. Sex distribution within the groups and the median age for each group are given in Table I.

Methods

Thyroglobulin antibodies were measured using two different methods:

I. A solid-phase immunosorbent radioassay based on the binding of homologous ^{125}I -labelled thyroglobulin to the antibodies which are then detected by their binding to anti-IgG antibodies covalently coupled to Sepharose as described in detail elsewhere (Ericsson et al., in press). All samples were assayed in duplicate at final dilutions of 1:5, 1:50 and 1:500. Sera with a binding of ^{125}I -Tg of more than 15% in the 1:5 dilution were regarded antibody positive.

Table I. Sex distribution and median age in the eight diagnostic groups.

Diagnosis	Males	Females	Age, years	
	No	No	Median	Range
Healthy women	--	80	48 and 53	
Healthy men	60	--	48	29-52
Atoxic goitre	8	39	50	9-78
Toxic diffuse goitre	8	45	50	17-73
Toxic nodular goitre	3	26	52	32-76
Toxic adenoma	3	16	50	28-70
Primary hypothyroidism	1	26	62	29-88
Thyroid carcinoma	14	39	58.5	17-90

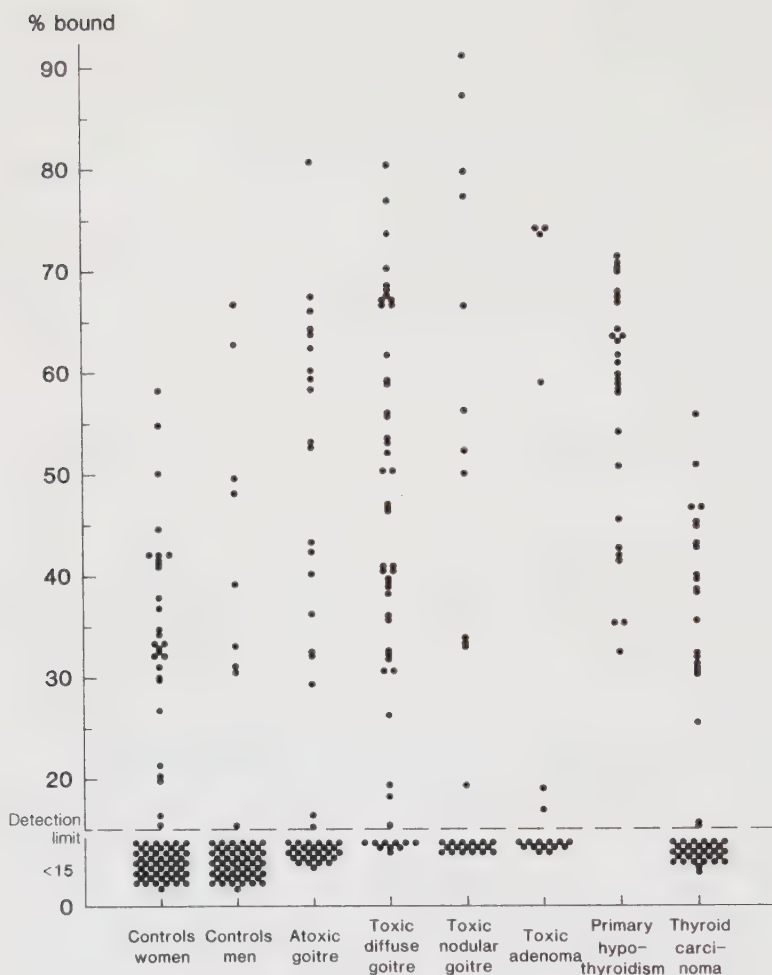


Fig. 1. Distribution of the total bound activity in the thyroglobulin antibody radioassay, in patients with different thyroid disorders and in healthy controls.

II. Tg-ab were also measured with a passive haemagglutination test using the Wellcome test (Thymune-T, Wellcome Research Laboratories, Beckenham, England). Sera with an antibody titre of 1:10 or more were regarded positive.

RESULTS

In Figure 1 the individual binding data with the radioassay are given. Table II lists the frequencies of Tg-ab positive sera in the

Table II. Distribution of thyroglobulin antibody positive sera among healthy individuals and patients with different thyroid disorders as measured with a solid-phase immunosorbent radioassay and a passive haemagglutination method.

Diagnosis		No	Tg antibody positive sera	
			RIA ¹	TRC ²
Healthy women		80	29 (36%)	0
Healthy men		60	9 (15%)	1 (2%)
Atoxic goitre	Male	8	6 (75%)	2 (25%)
	Female	39	14 (36%)	4 (10%)
Toxic diffuse goitre	Male	8	6 (75%)	2 (25%)
	Female	45	37 (82%)	7 (16%)
Toxic nodular goitre	Male	3	1 (33%)	0
	Female	26	11 (42%)	3 (12%)
Toxic adenoma	Male	3	1 (33%)	0
	Female	16	5 (31%)	0
Primary hypothyroidism	Male	1	1 (100%)	1 (100%)
	Female	26	26 (100%)	14 (54%)
Thyroid carcinoma	Male	14	5 (36%)	0
	Female	39	17 (44%)	8 (21%)

¹Radioimmunoassay, ²Passive haemagglutination test

different diagnostic groups as measured with the radioassay and the TRC test.

Control population

Among the 60 males, 9 (15%) were Tg-ab positive in the radioassay, as compared with only one individual in the TRC test. In the female group Tg-ab were found in 29/80 (36%) using the RIA test. All of the 29 serum samples were negative with the TRC test. The distribution of the binding for the positive samples was similar to that found in most patient groups.

Patients with thyroid disorders

All 27 patients with primary hypothyroidism were Tg-ab positive in

the RIA with a bound activity above 30%, which is higher than was found in any of the other groups. With the TRC test only 15 persons were Tg-ab positive. Only 4 of the 10 individuals with a morphological diagnosis of chronic thyroiditis were Tg-ab positive in the TRC test.

With the RIA, a high incidence of Tg-ab positive sera was also found among the patients with toxic diffuse goitre (81%), whereas the patients with toxic nodular goitre, toxic adenoma, atoxic goitre and thyroid carcinoma were Tg-ab positive at about the same frequency as the controls. All subjects with atoxic goitre, in whom there was a histologic or cytologic diagnosis of chronic thyroiditis, were Tg-ab positive in the RIA, but only 2/10 had detectable Tg-ab with the TRC test. In the thyroid carcinoma group, 7/22 (32%) of the patients who were Tg-ab positive with the RIA, demonstrated focal or diffuse lymphocytic infiltration of their thyroid glands. Two of these seven persons were also positive with the TRC test.

Of the 42 sera which were positive in the TRC test, 35 (83%) were also positive in the Tg-ab RIA with a titre of 1:50 or more. Seven sera (4%) were positive in the TRC test but negative with the RIA method.

DISCUSSION

In this study we found Tg-ab in 27% (36% of the women and 15% of the men) with the RIA method, despite the fact that the control group was selected from among healthy individuals, great care being taken to exclude the presence of thyroid disorders. The prevalence found here is higher than has been reported with other Tg-ab RIA. Peake et al. (1974) found Tg-ab in 4% of their control population including normal subjects and patients hospitalized with other than thyroid diseases. Date et al. (1980) found Tg-ab in 6% of healthy blood donors using a co-precipitation method, whereas Salabè et al. (1972) found an overall incidence of 15% in normal human sera.

The tanned red cell haemagglutination test or modifications thereof,

is the technique most frequently used to detect Tg-ab in human serum. With this method Hackett et al. (1960), in a mixed group of medical patients in Adelaide, without clinical evidence of thyroid disease, found Tg-ab in 18% (25.7% of the women and 10.6% of the men) when the cut-off titre was as low as 1:10. Dingle et al. (1966), estimated the prevalence of Tg-ab in a general practice in north-east England. They found Tg-ab in 16.2% of the women and 4.3% of the men, when a cut-off titre of 1:25 was chosen. Hill et al. (1961), using the same titre as cut-off, found Tg-ab in 7% of the women and 1.5% of the men in a patient population without thyroid disease. In the female population described by Borup Christensen et al. (1984), from which the 80 women in this study were recruited, 14/400 (3.5%) were Tg-ab positive with the TRC test. Despite the differences in prevalence in the control populations described above, the sex ratio for the Tg-ab positive individuals was about the same in all studies and in agreement with the sex ratio found in thyroid disease other than Hashimoto's thyroiditis in which an even higher female predominance is found (Roitt et al., 1960).

The low sensitivity of the TRC test is verified by the frequency of Tg-ab found in our patients with primary hypothyroidism. Only 56% of these patients were positive with the TRC test while 100% were positive in the RIA. The high prevalence of Tg-ab reported here with the use of the RIA methods is in agreement with other reports (Salabè et al., 1972; Peake et al., 1974; Feldt-Rasmussen et al., 1983).

In patients with toxic diffuse goitre (Graves' disease) Tg-ab were found in a high frequency, comparable to that observed in patients with primary hypothyroidism. With the RIA test 81% were Tg-ab positive compared to only 17% with the TRC test. Equal high prevalences (50-80%) have been described by others using radioimmunological methods (Mori & Kriss, 1971; Wilkin et al., 1980; Feldt-Rasmussen et al., 1983) and is comparable to those reported for anti-microsomal antibodies (Abreau et al., 1977; McGregor et al., 1980).

In all other groups (toxic nodular goitre, toxic adenoma, thyroid carcinoma and atoxic goitre) we found prevalences of Tg-ab comparable

to those found among the healthy controls. Peake et al. (1974), however, found Tg-ab in 8% of the sera from patients with thyroid disorders not considered to be of autoimmune etiology, which was higher than in their control population (4%). It is not known to what extent autoimmune mechanisms contribute to thyroid enlargement in sporadic non-toxic goitre. Lymphoid infiltration and the presence of germinal centres are, however, often seen on histological examination (Bastenie et al., 1972). A mild lymphocytic infiltration is also seen in most cases with papillary carcinoma, whereas a marked thyroiditis is observed in about 10% of these patients (Bastenie et al., 1972). Probably the thyroiditis in these patients represents the host's immune reaction to the tumour (LiVolsi & LoGerfo, 1981). Among our 21 patients with papillary carcinoma 6 had some degree of lymphocytic infiltration. Five of them were Tg-ab positive in the RIA.

The significance of the high incidence of Tg-ab in the apparently healthy individuals is controversial. From post-mortem examinations the reported prevalence of lymphocytic thyroiditis, including the focal type, have varied from 6.5 to 27.4% (Mortensen et al., 1955; Goudie et al., 1959; Williams & Doniach, 1962; Bastenie et al., 1967; Bastenie et al., 1972). Yoshida et al. (1978) found good correlation between the frequency of lymphocytic infiltration of the thyroid and that of positive serological tests. A correlation has also been shown to exist between the titre of circulating thyroid antibodies and the degree of lymphocytic infiltration of the thyroid (Persson, 1968; Bastenie et al., 1972).

The observed differences in prevalence of Tg-ab among the non-thyroid diseased individuals included in the various study groups, may be due to both methodological factors and to true differences in the populations studied. This radioassay is probably more sensitive than the others described (Salabè et al., 1972; Peake et al., 1974; Date et al., 1980) because of its capacity for working with a low dilution in the primary incubate (1:5), and a specific radioactivity of the tracer ten times higher than that of the other Tg-ab RIA:s. The sensitivity of this RIA may enable us to detect a higher proportion of the Tg-ab than has been possible before.

Gordin et al. (1974) found that most subjects with thyroid antibodies who were euthyroid had a symptomless autoimmune thyroiditis (SAT). Therefore, the high prevalence of Tg-ab found among apparently healthy individuals may indicate a more frequent occurrence of SAT than previously recognized. If so, this could be one explanation of the decrease in radioiodine uptake in the population, observed during the last 20 years (Ericsson & Thorell, 1981). To examine this assumption, follow-up studies in the same population will be needed.

REFERENCES

Abreau C M, Vagenakis A G, Roti E & Braverman L E (1977): Clinical evaluation of a hemagglutination method for microsomal and thyroglobulin antibodies in autoimmune thyroid disease. *Ann Clin Lab Sci* 7:73-78.

Bastenie P A, Neve P, Bonnyns M, Vanhaelst L & Chailly M (1967): Clinical and pathological significance of asymptomatic atrophic thyroiditis. A condition of latent hypothyroidism. *Lancet* 1:915-919.

Bastenie P A & Ermans A M (eds) (1972): *Thyroiditis and thyroid function. Clinical, morphological, and physiopathological studies.* Pergamon Press, Oxford.

Borup Christensen S, Ericsson U-B, Janzon L, Tibblin S & Trelle E (1984): The prevalence of thyroid disorders in a middle-aged female population, with special reference to the solitary thyroid nodule. *Acta Chir Scand* 150:13-19.

Date J, Feldt-Rasmussen U, Hyltoft Petersen P & Bech K (1980): An improved co-precipitation assay for determination of thyroglobulin antibodies. *Scand J Clin Lab Invest* 40:37-44.

Dingle P R, Ferguson A, Horn D B, Tubmen J & Hall R (1966): The incidence of thyroglobulin antibodies and thyroid enlargement in a general practice in north-east England. *Clin Exp Immunol* 1:277-284.

Doniach D, Bottazzo G F, Russell R C (1979): Goitrous autoimmune thyroiditis (Hashimoto's disease). *J Clin Endocrinol Metabol* 49:63-80.

Ericsson U-B & Thorell J I (1981): Decreased radioiodine uptake values without increase in urinary iodine excretion. *Acta Med Scand* 209:293-297.

Ericsson U-B, Larsson I & Thorell J I (In press): A new sensitive immunosorbent radioassay for the detection of circulating antibodies to polypeptide hormones and proteins. *Scand J Clin Lab Invest*.

Feldt-Rasmussen U, Perrild H, Bech K, Bliddal H, Date J, Høier Madsen M, Nordfang O, Ryder L P, Thomsen M, Kappelgaard E & Nielsen H (1983): Discrepancy between haemagglutination and radioimmunological techniques for measurement of serum thyroglobulin autoantibodies. *Allergy* 38:49-56.

Feldt-Rasmussen U (1983): Serum thyroglobulin and thyroglobulin autoantibodies in thyroid diseases. Pathogenic and diagnostic aspects. *Allergy* 38:369-387.

Gordin A, Saarinen P, Pelkonen R & Lamberg B-A (1974): Serum thyrotrophin and the response to thyrotrophin releasing hormone in symptomless autoimmune thyroiditis and in borderline and overt hypothyroidism. *Acta Endocrinol (Copenh)* 75:274-285.

Goudie R B, Anderson J R & Gray K G (1959): Complement-fixing anti-thyroid antibodies in hospital patients with asymptomatic thyroid lesions. *J Path Bact* 77:389-400.

Hackett E, Beech M & Forbes I J (1960): Thyroglobulin antibodies in patients without clinical disease of the thyroid gland. *Lancet* II: 402-404.

Hill O W (1961): Thyroglobulin antibodies in 1297 patients without thyroid disease. *Br Med J* 1:1793-1796.

Li Volsi V A & Lo Gerfo P (eds) (1981): *Thyroiditis*. Boca Raton, Florida, CRC Press p. 127.

Loeb P B, Drash A L & Kenny F M (1973): Prevalence of low-titer and "negative" antithyroglobulin antibodies in biopsy-proved juvenile Hashimoto's thyroiditis. *J Pediatr* 82:17-21.

McGregor A M, Petersen M M, McLachlan S M, Rooke P, Rees Smith B & Hall R (1980): Carbimazole and the autoimmune response in Graves' disease. *N Engl J Med* 303:302-307.

Mori T & Kriss J P (1971): Measurements by competitive binding radioassay of serum anti-microsomal and anti-thyroglobulin antibodies in Graves' disease and other thyroid disorders. *J Clin Endocrinol Metab* 33:688-698.

Mortensen J D, Woolner L B & Bennett W A (1955): Gross and microscopic findings in clinically normal thyroid glands. *J Clin Endocrinol Metab* 15:1270-1280.

Peake R L, Willis D B, Asimakis G K, Jr & Deiss W P, Jr (1974): Radioimmunologic assay for antithyroglobulin antibodies. *J Lab Clin Med* 81:907-919.

Persson P S (1968): Cytodiagnosis of thyroiditis. A comparative study of cytological, histological, immunological and clinical findings in thyroiditis, particularly in diffuse lymphoid thyroiditis. *Acta Med Scand suppl* 483.

Roitt I M & Doniach D (1960): Thyroid auto-immunity. Br Med Bull 16:152-158.

Salabè G B, Fontana S & Andreoli M (1972): Radioimmunoassay for human antithyroglobulin antibodies. I. The relationship between tanned cell haemagglutination and a double antibody technique. Hormones 3:1-13.

Whittingham S, Irwin J, Mackey I R, Marsh S & Cowling D C (1969): Autoantibodies in healthy subjects. Aust NZ Ann Med 18:130-134.

Wilkin T J, Swanson Beck J, Gunn A, Al Moussah M, Isles T E & Crooks J (1980): Autoantibodies in thyrotoxicosis: a quantitative study of their behavior in relation to the course and outcome of treatment. J Endocrinol Invest 3:5-14.

Williams E D & Doniach I (1962): The post-mortem incidence of focal thyroiditis. J Path Bact 83:255-264.

Yoshida H, Amino N, Yagawa K, Uemura K, Satoh M, Miyai K & Kumahara Y (1978): Association of serum antithyroid antibodies with lymphocytic infiltration of the thyroid gland: Studies of seventy autopsied cases. J Clin Endocrinol Metab 46:859-862.

The golden rule is that there are no golden rules

Bernard Shaw

